PROPOSAL: VOLUME I

DARPA – PREEMPT (HR001118S0017)
LEAD ORGANIZATION: EcoHealth Alliance (Other Nonprofit)
OTHER TEAM MEMBERS:
Duke NUS Medical School (Other Educational)
University of North Carolina (Other Educational)
Wuhan Institute of Virology (Other Educational)
USGS National Wildlife Health Center (Other Nonprofit)
Palo Alto Research Center (Large Business)

Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses



[redacted]

EcoHealth Alliance

24 March 2018

Dear Committee for DARPA PREventing EMerging Pathogenic Threats (PREEMPT),

Please accept the following proposal to the PREventing EMerging Pathogenic Threats (PREEMPT, HR001118S0017) program. The PI for this project is:

Dr. Peter Daszak
President, EcoHealth Alliance
460 W. 34th Street, 17th Floor
New York, NY 10001
212-380-4474
daszak@ecohealthalliance.org

Title: Project Defuse: Defusing the Threat of Bat-Borne Coronaviruses

Amount of the Requested Proposal: $14,209,245

Thank you for your time, and I look forward to hearing from you. If you have any questions, do not hesitate to call or email me.

Yours sincerely,

[redacted]

Section II A. EXECUTIVE SUMMARY

Technical Approach: Our goal is to defuse the potential for spillover of novel bat-origin high-zoonotic risk SARS-related coronaviruses in Asia. **In TA1** we will intensively sample bats at our field sites where we have identified high spillover risk SARSr-CoVs. We will sequence their spike proteins, reverse engineer them to conduct binding assays, and insert them into bat SARSr-CoV (WIV1, SHC014) backbones (these use bat-SARSr-CoV backbones, not SARS-CoV, and are exempt from dual-use and gain of function concerns) to infect humanized mice and assess capacity to cause SARS-like disease. Our modeling team will use these data to build **machine-learning genotype-phenotype models** of viral evolution and spillover risk. We will uniquely validate these with serology from previously-collected human samples via LIPS assays that assess which spike proteins allow spillover into people. We will build **host-pathogen spatial models** to predict the bat species composition of caves across Southeast Asia, parameterized with a full inventory of host-virus distribution at our field test sites, three caves in Yunnan Province, China, and a series of unique global datasets on bat host-viral relationships. By the end of Y1, we will create a prototype app for the warfighter that identifies the likelihood of bats harboring dangerous viral pathogens at any site across Asia.

In TA2, we will evaluate two approaches to reduce SARSr-CoV shedding in cave bats: **(1) Broadscale immune boosting**, in which we will inoculate bats with immune modulators to upregulate their innate immune response and downregulate viral replication; **(2) Targeted immune boosting**, in which we will inoculate bats with novel chimeric polyvalent recombinant spike proteins plus the immune modulator to enhance innate immunity against specific, high-risk viruses. We will trial inoculum delivery methods on captive bats including a novel automated aerosolization system, transdermal nanoparticle application and edible adhesive gels. We will use stochastic simulation modeling informed by field and experimental data to characterize viral dynamics in our cave test sites, maximize timing, inoculation protocol, delivery method and efficacy of viral suppression. The most effective biologicals will be trialed in our test cave sites in Yunnan Province, with reduction in viral shedding as proof-of-concept.

Management Approach: Members of our collaborative group have worked together on bats and their viruses for over 15 years. The lead organization, EcoHealth Alliance, will oversee all work. EHA staff will develop models to evaluate the probability of specific SARS-related CoV spillover, and identify the most effective strategy for delivery of both immune boosting and immune targeting inocula. Specific work will be subcontracted to the following organizations:

- Prof. Baric, Univ. N. Carolina, will lead targeted immune boosting work, building on his two-decade track record of reverse-engineering CoV and other virus spike proteins.
- Prof. Wang, Duke-Natl. Univ. Singapore, will lead work on broadscale immune boosting, building on his group's pioneering work on bat immunity.
- Dr. Shi, Wuhan Institute of Virology will conduct viral testing on all collected samples, binding assays and some humanized mouse work.
- Dr. Rocke, USGS National Wildlife Health Center will optimize delivery of immune modulating biologicals, building on her vaccine delivery work in wildlife, including bats.
- Dr. Unidad, Palo Alto Research Center will lead development of novel delivery automated aerosolization mechanism for immune boosting molecules.

We are requesting $14,209,245 total funds for this project across 3.5 project years.

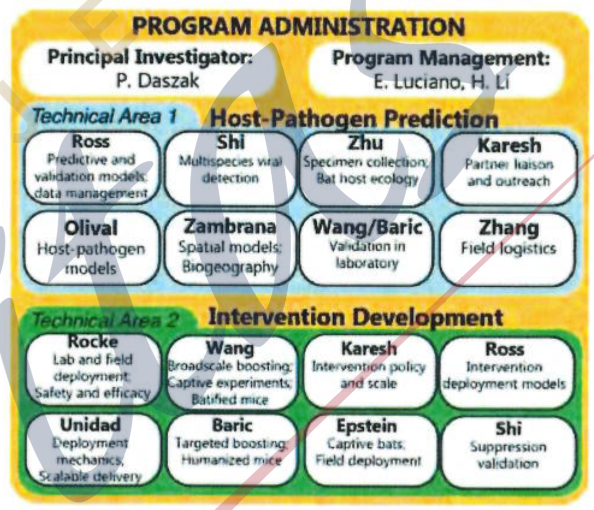
Section II C. GOALS AND IMPACT

Overview: The overarching goals of DEFUSE are to:

- **Identify and model spillover risk** of novel SARS-related coronaviruses (SARSr-CoVs) in Asia.
- **Design and demonstrate proof-of-concept** that upregulating the naturally low innate immunity of bats (**broadscale immune boosting**) and targeting high risk SARSr-CoVs in particular (**targeted immune boosting**) will transiently reduce spillover risk.

Our strategy to reduce risk of viral emergence from bats will protect the warfighter within USPACOM, and will be scalable to other regions and viruses (Ebola, Henipaviruses, rabies).

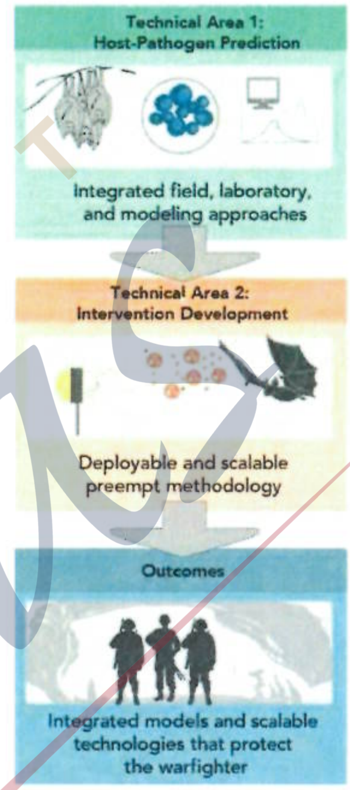
Innovation and uniqueness:



Bats harbor more emerging zoonoses than any other group of mammals, are ubiquitous, abundant, and often overlooked. However, other than PPE, there is no available technology to reduce exposure risk to novel CoVs from bats, and no effective therapeutics or countermeasures. SARSr-CoVs are enzootic in Asian[1-3], African[4], and European bats[5,6] that roost in caves but forage widely at night, shedding virus in their feces and urine. We have now published direct evidence of spillover of novel SARSr-CoVs into people in Yunnan Province, China, close to a cave complex where we have isolated strains that produce SARS-like disease in humanized mice but don't respond to antibody treatment or vaccination. These viruses are a clear-and-present danger to our military and to global health security because of their circulation and evolution in bats and periodic spillover into humans.

EcoHealth Alliance (EHA) leads the world in predictive models of viral emergence. We will use machine-learning models of spillover hotspots, host-pathogen spatial and genotype-phenotype mapping, and unique datasets to validate and refine hotspot risk maps of viral emergence. We have shown that dampened innate immunity in bats allows them to carry otherwise lethal viruses, likely as an adaptation to the physiologic stress of flight. We will design strategies like small molecule RIG-like receptor (RLR) or Toll-like receptor (TLR) agonists, to upregulate bat immunity, and suppress viral replication, thereby significantly reducing viral shedding and spillover **(broadscale immune boosting)**. We will complement this by coupling agonist treatments with SARSr-CoV recombinant spike proteins to boost pre-existing adaptive immune response adult bats against specific, high-risk SARSr-CoVs **(targeted immune boosting)**. We will design novel delivery and automated application methods, based on our previous work on wildlife vaccines, to reduce hazard during deployment.

Technical Area 1



Our strategy begins by a complete inventory of bats and their SARSr-CoVs at our intervention test site cave complex in Yunnan, China that harbors bats with high-risk SARSr-CoVs. We will collect data from three caves in that system (one is our intervention test site and two control sites) on: monthly bat abundance and diversity, viral prevalence and diversity, individual bat viral load and host physiological markers; genomic characterization of low- and high-risk SARSr-CoV strains among bat species, sexes, and age classes; satellite telemetry and mark-recapture data on bat home range and inter-cave movement; and monitoring of daily, weekly and seasonal changes in bat populations. We will use stochastic neural networks to build joint species distribution models (JSDM) to predict bat species composition of caves, and high-risk SARSr-CoV diversity across S. China, South and SE Asia. These will be parameterized with EHA's database of bat host-viral relationships and estimates of zoonotic viral richness per bat species[7]; biological inventory data on all bat caves in Southern China; the full SARSr-CoV inventory from our cave test sites in Yunnan; and species distribution data for all bats. We will test and validate viral diversity predictions using data from >10,000 previously collected bat samples from 6 Asian countries under our USAID-funded PREDICT project. We will produce a prototype app for the warfighter to identify the risk of bats harboring dangerous viruses at a site. **This 'spatial viral spillover risk' app** will be field-deployable and updated real-time with surveillance data, to ground-truth and fine-tune predictions.

To characterize spillover risk of SARSr-CoV quasispecies (QS), the Wuhan Institute of Virology team (WIV) will test bat fecal, oral, and blood samples for SARSr-CoVs by PCR. We will collect viral load data from fresh fecal pellets. SARSr-CoV spike proteins will be sequenced, viral recombination events identified, and isolates used to identify strains that can replicate in human cells. The Univ. N. Carolina (UNC) team will reverse-engineer spike proteins of a large sample of high- and low-risk viruses for further characterization. This will effectively freeze the QS we analyze at t=0. These QS_0 strain viral spike glycoproteins will be synthesized, and those binding to human cell receptor ACE2 will be inserted into SARSr-CoV backbones (non-DURC, non-GoF), and inoculated into humanized mice to assess capacity to cause SARS-like disease, efficacy of monoclonal therapies, the inhibitor GS-5734[8] or vaccines against SARS-CoV[8-12].

We will use these data to **build machine-learning <u>genotype-to-phenotype Bayesian network models</u> of viral evolution and host jump risk**. These will predict the capacity of QS_0 strains to infect human cells based on genetic traits and experimental assays above. Using data on diversity of spike proteins, recombinant CoVs, and flow of genes via bat movement and migration, we will estimate evolutionary rates, rates of recombination, and capacity to generate novel strains capable of human infection. Finally, virus-host relationship and bat home range data will be used to estimate spillover potential - extending models well beyond our field sites. We will **validate model predictions of host jump risk** by **1)** conducting further spike

protein-based binding and cell culture experiments, and **2)** identifying whether designated high-risk SARSr-CoV strains have already spilled over into people near our bat cave sites. Our preliminary work shows ~3% seroprevalence to bat SARSr-CoVs in people at this site[13]. We will test these previously collected human sera (n>2000) for presence of antibodies to the high- and low-risk SARSr-CoVs identified by our modeling, using Luciferase immunoprecipitation system (LIPS) assays we design against the SARSr-CoVs identified in this project[14].

Technical Area 2

In TA2, we will **develop scalable approaches to suppress SARSr-CoVs within bat reservoir species, to reduce the likelihood of virus transmission into humans.** We will evaluate two approaches to defuse spillover potential: **1) Broadscale immune boosting:** we will apply immune modulators like bat interferon and TLR agonists to up-regulate bat innate immunity and suppress viral replication and shedding; **2) Targeted immune boosting:** we will apply polyvalent chimeric recombinant SARSr-CoV spike proteins in the presence of broadscale immune boosting treatments to boost immune memory and suppress specific SARSr-CoVs.

Both TA2 lines of work will run parallel beginning Yr 1. Prof. Wang (Duke-Natl. Univ. Singapore – Duke-NUS) will lead the broadscale immune boosting work, building on his pioneering work on bat immunity[15], including identifying weakened functionality of innate immunity factors like STING, a central DNA-interferon (IFN) sensing molecule, that may allow bats to maintain an effective, but not over-response to viruses[16], and IFNA, which is constitutively expressed without stimulation[17]. We will trial the following, concurrently and competitively, for efficacy and scalability: **i)** Activating TLR/RLR pathways to induce IFN induction, e.g. polyIC or 5'ppp-dsRNA. A similar strategy has been demonstrated in a mouse model for SARS-CoV[18,19]; **ii)** Universal bat interferon. Interferon has been used clinically in people, e.g. against filoviruses[20], and replication of SARSr-CoV is sensitive to interferon[21]; **iii)** Boosting bat IFN by blocking negative regulators. Bat IFNα is constitutively expressed but cannot be induced to a high level[17]. We will use CRISPRi to identify potential negative regulators and screen for compounds targeting this gene; **iv)** Activating dampened IFN production pathways via DNA-STING-dependent and ssRNA-TLR7-dependent pathways. Mutant bat STING restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence[16]. We will directly activate the pathways downstream of STING/TLR7, to promote viral clearance; **v)** Inoculating crude CoV fragments to upregulate innate immune responses to specific CoVs – a partial step towards the targeted immune boosting work below.

Prof. Baric (UNC) will lead the targeted immune boosting work. We will develop recombinant chimeric spike-proteins[22] from known SARSr-CoVs, and those characterized by DEFUSE. Using details of SARS S protein structure and host cell binding[23] we will sequence, reconstruct and characterize spike trimers and receptor binding domains of SARSr-CoVs, incorporate them into nanoparticles or raccoon poxvirus-vectors for delivery to bats[10,24-27]. In combination with immune-boosting small molecules, we will use these to boost immune memory in adult bats previously exposed to SARSr-CoVs, taking the best candidate forward for field-testing. Recombinant S glycoprotein-based constructs with immunogenic blocks from across group 2B SARSr-CoVs should induce broadscale adaptive immune responses that reduce heterogeneous virus burdens in bats and transmission risk to people[28,29]. Innate immune damping is highly conserved in all bat species tested so far. We will use the unique Duke-NUS

Asian cave bat (*Eonycteris spelaea*) breeding colony to conduct initial proof-of-concept tests, extended to small groups of wild-caught *Rhinolophus sinicus* bats at WIV.

A novel delivery method for our immune boosting molecules will be developed and implemented by Dr. Rocke at the USGS National Wildlife Health Center (NWHC) who has previously developed animal vaccines through to licensure[30]. Using locally acquired insectivorous bats[31,32], we will assess delivery vehicles and methods including: 1) transdermally applied nanoparticles; 2) sticky edible gels that bats mutually groom and consume; 3) aerosolization via prototype sprayers (Dr. Unidad, PARC) designed for cave settings; and 4) automated sprays triggered by timers and movement detectors at critical cave entry points We have extensive preliminary data on these techniques for wildlife, including vaccinating bats against rabiesin the lab[31], successful delivery, consumption and spread in wild vampire bats. We will use the NWHC captive bat colony and wild bats in US caves to trial delivery vehicles using the biomarker rhodamine B (which fluorescently marks hair on consumption) to assess uptake. **The most optimal deployment approaches will be tested on wild bats at our test cave sites in Yunnan, using the most effective immune modulation preparations.** Bat populations from experimental and control caves will be surveyed longitudinally for viral load before and after deployment trials. EHA has had unique access to these sites for ~10 years. In DEFUSE Yr1, we will seek permission for experimental trials from collaborators at the Yunnan Forestry Department and Center for Disease Control, following our proven track record of rapidly obtaining IACUC and DoD ACURO approval for animal research. We will **model optimal strategies to maximize treatment efficacy** for TA2, using stochastic simulation modeling of viral circulation dynamics at our sites, informed by field and experimental data. We will estimate frequency and population coverage required for our intervention, and model the time period of viral suppression, until re-colonization or evolution leads to return of a high-risk SARSr-CoV.

Deliverables:

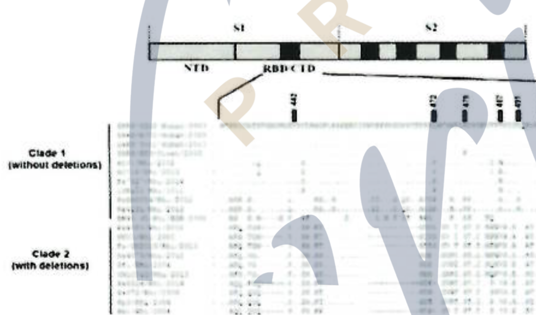
- Open source models and App identifying geographical and host-specific risk of spillover for novel SARSr-CoVs
- Experimentally validated genotype-phenotype models of spillover for viral strains.
- Proven technology to modulating bat innate immunity to reduce viral shedding
- Tested and validated delivery mechanism for bat cave usage including vaccines in other bat host-pathogen systems (e.g. rabies, WNS).
- Proof-of-concept approach to transiently reducing viral shedding in wild bats that can be adapted for other systems including Ebola virus.

Section II D. TECHNICAL PLAN

Technical Area I:

Choice of site and model host-virus system. For the past 14 years, our team has conducted CoV surveillance in bat populations across S. China, resulting in >180 unique SARSr-CoVs in ~10,000 samples (>5% prevalence, including multiple individuals harboring the same viral strains)[2,21,33] and a per-bat species prevalence up to 10.9%. Bat SARSr-CoVs are genetically diverse, especially in the S gene, and most are highly divergent from SARS-CoV. However, our test cave site in Yunnan Province, harbors a quasispecies (QS) population assemblage that contains all the genetic components of epidemic SARS-CoV[34]. We have isolated three strains there (WIV1,

WIV16 and SHC014) that unlike other SARSr-CoVs, do not contain two deletions in the receptor-binding domain (RBD) of the spike, have far higher sequence identity to SARS-CoV (**Fig. 1**), use human ACE2 receptor for cell entry, as SARS-CoV does (**Fig. 2**), and replicate efficiently in various animal and human cells[2,3,33-35], including primary human lung airway cells, similar to epidemic SARS-CoV[11,12]. Chimeras (recombinants) with these SARSr-CoV S genes inserted into a SARS-CoV backbone, and synthetically reconstructed full length SHCO14 and WIV1 cause SARS-like illness in humanized mice (mice expressing human ACE2), with clinical signs that are not reduced by SARS-CoV monoclonal antibody therapy or vaccination[11,12]. People living up to 6 kilometers from our test cave have SARSr-CoV antibodies (~3% seroprevalence)[13], suggesting active spillover. These data, phylogeography of SARSr-CoVs, and coevoutionary analysis of bats and their CoVs (unpubl.), suggest that bat caves in SW China, and *Rhinolophus* spp. bats are the likely origin of the SARS-CoV clade, and are **a clear-and-present danger for the emergence of a SARSr-CoV from the current QS.** The *Rhinolophus* spp. bats that harbor these viruses occur across Asia, Europe, and Africa. **Thus, while DEFUSE fieldwork will focus on high-risk sites in S. China, our approach to reduce the risk of these viruses spilling over is broadly applicable across four combatant command regions (PACOM, CENTCOM, EUCOM, AFRICOM).**



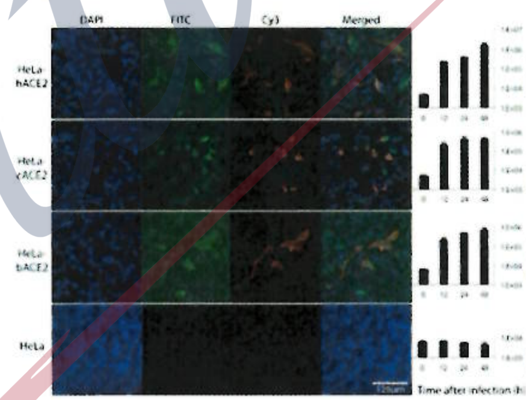


***Fig. 1** (top left): Alignment of amino acid sequences of the spike protein receptor-binding motif of SARSr-CoVs and SARS-CoV[34]. Numbered amino acid residues are responsible for interaction with human ACE2[36].* ***Fig. 2** (top right): Bat SARSr-CoV WIV1 replicates efficiently in HeLa cells expressing human, civet and bat ACE2[2].*

Full inventory of bat SARSr-CoV QS at our test cave sites, Yunnan, China. To provide data to train and validate our modeling, and as baseline for our immune modulation trial (TA2), DEFUSE fieldwork will target the high-risk cave site in Yunnan Province, SW China (**Fig. 4, red triangle**) where we will conduct our field trial, and where we have previously identified and isolated high-risk SARSr-CoVs[2,11,33,34]. At three cave sites (one designated for our trial, two as controls), we will determine the baseline QS_0 risk of SARSr-CoV spillover. We will conduct longitudinal surveillance of bat populations to detect and isolate SARSr-CoVs, determine changes in viral prevalence over time, and measure bat population demographics and movement, definitively characterizing their SARSr-CoV host-viral dynamics. Field data will allow us to test the accuracy of our model predictions and compare efficacy of lab animal models with field trials. Our preliminary data (**Table 1**) demonstrate that *R. sinicus*, *R. ferrumequinum,* and *R. affinis* (which co-roost at our test site) are primary reservoirs of SARSr-CoV and the only reservoirs of three high-risk strains (WIV1, WIV16, SHC014), with *Hipposideros* and *Myotis* spp. playing an

insignificant (<1% prevalence) role in viral dynamics. We will capture *Rhinolophus spp*. bats using harp traps and mist nets during evening flyout, collect rectal, oral, and whole blood samples (×**2** per bat) using sterile technique to avoid cross-contamination, and take 2-mm wing tissue punch biopsies for host DNA bar-coding, host ACE2 receptor gene sequencing (interface site – 3 individuals per species), and cophylogeny analyses. Bats will be subcutaneously microchipped (PIT tag), and morphological and physiological data (age class, body weight, reproductive status etc.).

Species	n	SARSr-CoV prevalence
Rhinolophus sinicus	1036	10.9%
R. ferrumequinum	191	6.3%
R. affinius	518	1.2%

In Phase I we will **sample 60 bats each of** *R. sinicus*, *R. ferrumequinum*, and *R. affinis*, (**180** bats per cave) **every three months** non-destructively for **18 months** from our three cave sites. Given ~6-9% prevalence (n=3,304) of SARSr-CoVs in *Rhinolophus* spp. at our sites, this sample size would allow detection of 10% fluctuation in viral prevalence among sampling periods and caves. During the 2 months per quarter without physical bat trapping we will collect fresh fecal pellets by placing clean $2m^2$ polyethylene sheets beneath roosting bats[37]. *Rhinolophus* spp. have a 7-week gestation period, spring birthing, and aggregate during mating periods. Our monthly sampling strategy will collect adequate data to parameterize stochastic simulation models, and cover two mating and gestation periods to assess life-history driven changes in viral prevalence and immune marker (e.g. interferon) levels. We will conduct **pre- and post-intervention sampling** (biweekly fecal pellet sampling for 4 months, and 10 male and 10 female bats per species tested every 2 weeks post-intervention for 4 months, prior to- and post-deployment) **to monitor SARSr-CoV QS and bat immune status changes in test and control site bats during Phase II (TA2)**. Immune status can be followed in individual bats due to the relatively small roost sizes in these caves and our individual marking of captured bats. We will be assess immune status using nanostring immune profiling panels validated during captive bat studies at Duke NUS. We will use infrared spotlights and digital infrared imaging to record the number and species of bats above each plastic sheet and fecal pellets will be genetically barcoded to confirm species identification[38]. Samples will be preserved in viral transport medium, immediately frozen in liquid nitrogen dry shippers, and transported to partner laboratories with maintained cold chain and under strict biosafety protocols. PIT tag readers and weatherproof thermal imaging IR cameras mounted at each cave entrance will passively monitor temporal roost site fidelity, rates of inter-cave movement, and daily fluctuation in bat population[39]. ICARUS satellite transmitters (1g) will be attached to 12 *Rhinolophus* spp. bats from each study roost (36 bats total) to determine nightly foraging dispersal patterns (https://icarusinitiative.org). Telemetry and PIT tag data will be used to calculate home range, degree of mixing among roosts, and parameterize dynamic models. **Study caves will be surveyed using portable LiDAR technology**[40-42], to give a 3-D image of roost areas and data on species composition for targeting of immune modulation treatments in TA2 (**Fig. 3**). Sampling quotas will be adjusted based on lab and model results to optimize viral detection.

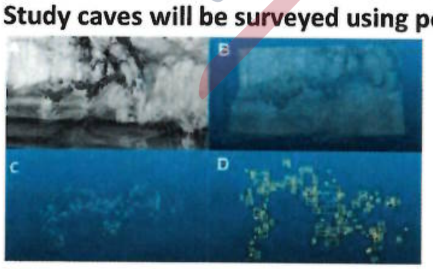
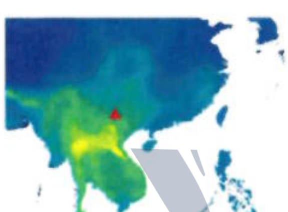


Fig. 3: Light Detection and Ranging (LiDAR) scanning to characterize caves and quantify number of individual bats roosting in clusters: A) LiDAR system takes a 360° omnidirectional photo of clustered bats, B)

photo converted to 3-D point cloud, C) non-bat points, based on laser return intensity removed, D) automated counting algorithm counts individual bats. Figure from[41].

Our team has more than 50 years collective experience in safe and humane handling of bats for biological sampling. This project will operate under appropriate IACUC/ACURO and PPE guidelines. EHA has several ongoing DTRA-supported projects, has obtained ACURO approval for animal research from the DoD, and currently maintains IACUC protocols through Tufts University (EHA staff are adjunct faculty), which we will use for DEFUSE IACUCs. IACUCs already approved for lab/field work at Duke-NUS, UNC, NWHC, and WIV, will be modified for DEFUSE.

Predictive models of high-risk sites and bat species across Asia. We will build models that predict bat and viral diversity and spillover risk across Asia to enable warfighters and planners to assess risk and necessity for intervention deployment (TA2). We will combine regional-scale joint species distribution models (JSDM), machine-learning host-virus association models, and non-parametric viral richness estimators to respectively predict the composition of bat communities in caves across Asia, host range for key viral clades, and as-of-yet unsampled viral diversity. We will use a stochastic feedforward neural network to implement JSDMs that are effective at multiple scales with incomplete observations (as occurs for bats and their viruses), and that account for bat species co-occurrence driven by environment or evolution[43]. We will fit our JSDM to biological inventory data on over 200 caves in the region[44], to physiologically relevant bioclimatic variables (BIOCLIM)[45], open source topographic data, and proxies for subterranean habitat such as ruggedness and habitat heterogeneity. As in previous work[46], we will refine these models with regional-scale environmental variables (land-use, distance to roads, etc.) and cave-specific variables (cave length, availability of roosting area, entrance dimensions, cave complexity etc.). We will validate them using independent bat occurrence estimates and observations[47,48], and use EHA's unique database of all known host-virus relationships to extend predictions of bat CoV diversity and host range[7] (**Fig. 4**). We will use generalized additive host trait predictive models and machine-learning algorithms (BRT, random forest)[49] with non-parametric estimators to predict SARSr-CoV diversity in the QS of each bat species[50], and assess viral discovery rates in real time through sampling (**Fig. 5**).



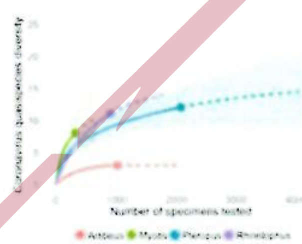


***Fig. 4**: Predictive map of zoonotic viral diversity in bats for China and SE Asia (yellow=more viruses), based on all known mammal host-viral relationships[7]. Our Yunnan test cave site is labeled (red asterisk).* ***Fig. 5**: CoV QS diversity estimates (dashed line with 95% confidence intervals) based on PREDICT sampling data (solid line) for four bat genera.*

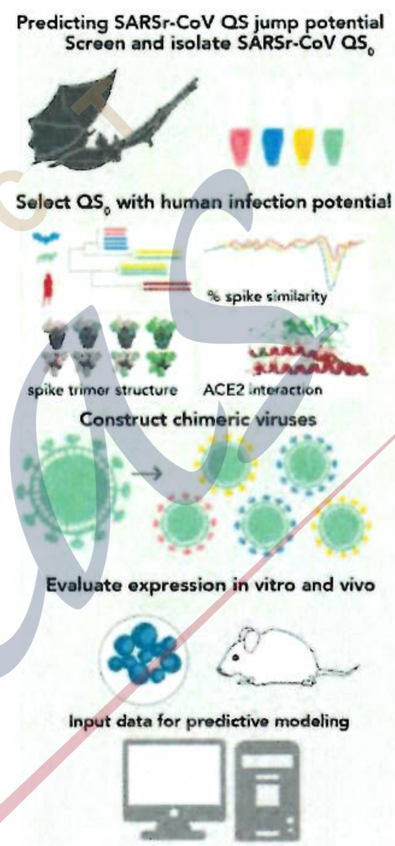
To extend the geographic scope of predictive models, we will include data from >1800 viral detections (CoVs and others) from >10,000 individual bat samples in 6 Asian countries (NIAID- and USAID PREDICT-funded). For species composition and viral presence predictions, we will validate models against a 20% validation subset of data, and field data.

Prototype app for the warfighter. Drawing on experience building applications for data collection and analysis for DoD (e.g. https://flirt.eha.io/, https://eidr-connect.eha.io/, https://mantle.io/grrs), we will produce a prototype **'spatial viral spillover risk' app** for the

warfighter that identifies probability of dangerous viral pathogens spilling over from bats at a site. We will use outputs from our spatial risk modeling, observed and predicted host-viral associations, open-source species and pathogen ontologies, and app-directed crowd-sourced echolocation data to ground-truth and fine-tune its predictive capacity. This app will be updated in Y2 and Y3 to incorporate additional risk data from host-virus binding assays and SARSr-CoV surveys. We will use EHA's risk-ranking algorithms (https://ibis.eha.io/) to display critical areas of high risk based on geolocation features, recency of information, host and pathogen characteristics. The app will collect user GPS location data and preload bat species distribution and community composition estimates from our JSDMs. These will be refined with real-time surveillance data collected without the need to enter cave sites using mobile phone-enabled high-frequency microphones for bat detection[51], validated and trained with reference acoustic calls using convolutional neural networks[52]. Identified bat species will be automatically linked with viral diversity data from EHA's host-pathogen database and SARSr-CoV data from DEFUSE to deliver high-risk pathogen lists, displayed as pathogen-centric, bat-centric, or map-centric views, with proactive alerts when critical information is received. All code modules will be available and documented on GitHub (https://github.com/ecohealthalliance/). This technology will improve overall situational awareness of existing and novel infectious agents found in bats, allowing DoD personnel to quickly identify areas high spillover risk sites and rapidly deploy resources to respond to and mitigate their impact preemptively when necessary.

SARSr-CoV QS detection, sequencing, and recovery. We will screen samples for SARSr-CoV nucleic acid using our pan-CoV consensus one-step hemi-nested RT-PCR assay targeting a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs[1,53], and specific assays for known SARSr-CoVs[2,21,33,34]. PCR products will be gel purified, sequenced and qPCR performed on SARSr-CoV-positive samples to determine viral load. Full-length genomes or S genes of all SARSr-CoVs will be high-throughput sequenced followed by genome walking[2,3,34]. We will analyze the S gene for its ability to bind human ACE2 by Biocore or virus entry assay. *Synthesis of Chimeric Novel SARSr-CoV QS:* We will commercially synthesize SARSr-CoV S glycoprotein genes, designed for insertion into SHC014 or WIV16 molecular clone backbones (88% and 97% S-protein identity to epidemic SARS-Urbani). These are BSL-3, not select agents or subject to P3CO (they use bat SARSr-CoV backbones which are exempt) and are pathogenic to hACE2 transgenic mice. Different backbone strains increase recovery of viable viruses identification of barriers for RNA recombination-mediated gene transfer between strains[34]. Recombinant viruses will be recovered in Vero cells, or in mouse cells over-expressing human, bat or civet ACE2 receptors to support cultivation of viruses with a weaker RBD-human ACE2 interface. *Recovery of Full length SARSr-CoV:* We will compile sequence/RNAseq data from a panel of closely related strains (<5% nucleotide variation) and compare full length genomes, scanning for unique SNPs representing sequencing errors[54-56]. Consensus candidates genomes will be synthesized commercially (e.g. BioBasic), using established techniques and genome-length RNA and electroporation to recover recombinant viruses[28,57].

Predicting strain-specific SARSr-CoV spillover risk. We will combine detailed experimental characterization of QS_0 at our test cave sites with state-of-the-art genotype-phenotype Bayesian network models. This will enable us to predict the jump probability of future QS that emerge with unique genetic recombinations. Our models will be parameterized with experimental data from a series of assays on the S genes of bat SARSr-CoVs (**Fig. 6, right**), with experimental and modeling work flowing together in iterative steps. Our prior data will act as baseline to parameterize spillover risk modeling[11,12,29,58]. This will be supplemented by characterization of isolated viruses under DEFUSE (at WIV), **approximately 15-20 bat SARSr-CoV spike proteins/year** (at UNC, WIV), and >180 bat SARSr-CoV strains sequenced in our prior work and not yet examined for spillover potential. All experiments will be performed in triplicate and data fed to models in real time:



Experimental assays of SARSr-CoV QS jump potential (Fig. 6, right). *Pre-screening via structural protein modeling, mutation identification, and pseudovirus assays:* Viral entry is the major species restriction preventing spillover of SARSr-CoVs[29,58]. To select QS for further characterization we will first use structural modeling of SARSr-CoV S protein binding to ACE2 receptors[59,60]. Mutations in the RBD[29,58,61,62], and host protease proteolytic processing of the S glycoprotein[63-65], regulate SARSr-CoV cell entry and cross-species infectivity. Mismatches in the S-RBD-ACE2 molecules or S proteolytic processing will prevent cell entry of SARS-CoV[29,58] and QS with these mismatches will be deprioritized. Single amino acid variations could dramatically alter these phenotypes and we will evaluate the impact of low abundant, high consequence micro-variation in the RBD using RNAseq to identify low abundant QS variants encoding mutations relevant to ACE2 binding. We will conduct *in vitro* pseudovirus binding assays, using established techniques[2], and live virus binding assays (at WIV to prevent delays and unnecessary dissemination of viral cultures) for isolated strains. Initial model predictions based on these data inputs will be used to guide strain selection for further characterization. *In vitro testing of chimeric viruses:* All chimeric viruses will be sequence verified and evaluated for: **i**) ACE2 receptor usage across species *in vitro*, **ii**) growth in primary HAE, **iii**) sensitivity to broadly cross neutralizing human monoclonal antibodies that recognize unique epitopes in the RBD[66,67]. Should some isolates prove highly resistant to our mAB panel, we will evaluate cross neutralization against a limited number of human SARS-CoV serum samples from the Toronto outbreak. Chimeric viruses that encode novel S genes with spillover potential will be used to identify SARSr-CoV strains for recovery as full genome length viable viruses. *In vivo pathogenesis:* Groups of 10 animals will be infected intranasally with 1.0×10^4 PFU of each vSARSr-CoV, clinical signs (weight loss, respiratory function, mortality, etc.) followed for 6 days

p.i., and sacrificed at day 2 or 6 p.i. for virologic analysis, histopathology and immunohistochemistry of the lung and for 22-parameter complete blood count (CBC) and bronchiolar alveolar lavage (BAL). *Validation with full-length genome QS:* We will validate results from chimeric viruses by re-characterizing full-length genome versions, testing whether backbone genome sequence alters full length SARSr-CoV spillover potential. QS for full-genome characterization will be selected to reflect strain differences in antigenicity, receptor usage, growth in human cells and pathogenesis. We will test growth in primary HAE cultures and *in vivo* in hACE2 transgenic mice. We anticipate recovering ~3-5 full length genome viruses/yr. *Testing Synthetic Modifications:* We will synthesize QS with novel combinations of mutations to determine the effects of specific genetic traits and the jump potential of future and unknown recombinants. *RBD deletions:* Small deletions at specific sites in the SARSr-CoV RBD alter risk of human infection. We will analyze the functional consequences of these RBD deletions on SARSr-CoV hACE2 receptor usage, growth in HAE cultures and *in vivo* pathogenesis. First, we will delete these regions, sequentially and in combination, in SHC014 and SARS-CoV Urbani, anticipating that the introduction of deletions will prevent virus growth in Vero cells and HAE[58]. In parallel, we will evaluate whether RBD deletion repair restores the ability of low risk strains to use human ACE2 and grow in human cells. *S2 Proteolytic Cleavage and Glycosylation Sites:* After receptor binding, a variety of cell surface or endosomal proteases[68-71] cleave the SARS-CoV S glycoprotein causing massive changes in S structure [72] and activating fusion-mediated entry[64,73]. We will analyze all SARSr-CoV S gene sequences for appropriately conserved proteolytic cleavage sites in S2 and for the presence of potential furin cleavage sites[74,75]. SARSr-CoV S with mismatches in proteolytic cleavage sites can be activated by exogenous trypsin or cathepsin L. Where clear mismatches occur, we will introduce appropriate human-specific cleavage sites and evaluate growth potential in Vero cells and HAE cultures. In SARS-CoV, we will ablate several of these sites based on pseudotyped particle studies and evaluate the impact of select SARSr-CoV S changes on virus replication and pathogenesis. We will also review deep sequence data for low abundant high risk SARSr-CoV that encode functional proteolytic cleavage sites, and if so, introduce these changes into the appropriate high abundant, low risk parental strain. *N-linked glycosylation:* Some glycosylation events regulate SARS-CoV particle binding DC-SIGN/L-SIGN, alternative receptors for SARS-CoV entry into macrophages or monocytes[76,77]. Mutations that introduced two new N-linked glycosylation sites may have been involved in the emergence of human SARS-CoV from civet and raccoon dogs[77]. While the sites are absent from civet and raccoon dog strains and clade 2 SARSr-CoV, they are present in WIV1, WIV16 and SHC014, supporting a potential role for these sites in host jumping. To evaluate this, we will sequentially introduce clade 2 disrupting residues of SARS-CoV and SHC014 and evaluate virus growth in Vero cells, nonpermissive cells ectopically expressing DC-SIGN, and in human monocytes and macrophages anticipating reduced virus growth efficiency. We will introduce the clade I mutations that result in N-linked glycosylation in rs4237 RBD deletion repaired strains, evaluating virus growth efficiency in HAE, Vero cells, or nonpermissive cells ± ectopic DC-SIGN expression[77]. *In vivo*, we will evaluate pathogenesis in transgenic hACE2 mice. *Low abundance micro-variations:* We will structurally model and identify highly variable residue changes in the SARSr-CoV S RBD, use commercial gene blocks to introduce these changes singly and in combination into the S glycoprotein gene of the low risk, parental strain and test ACE2 receptor usage, growth in HAE and *in vivo* pathogenesis.

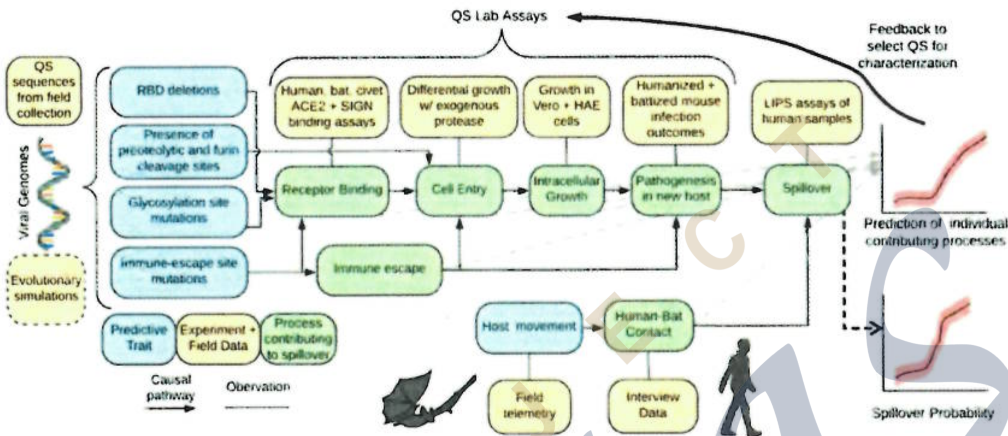


***Fig. 7**: A simplified directed graph of a Bayesian network model representing the causal relationships between input data, modeled processes, and outputs.*

Network machine-learning to predict spillover potential of high-risk SARSr-CoV strains. We will use experimental data from above to **build genotype-phenotype models of bat SARSr-CoV spillover potential**. We will use Bayesian Network Models (BNM), fit via MCMC methods[78] to predict spillover risk based on bat SARSr-CoVs genotype data (presence of deletions in RBD, proteolytic binding and glycosylation sites etc.) and the ecological traits of hosts - integrating data on multiple, interacting processes and QS spillover potential to generate overall spillover probabilities. The Bayesian approach will allow us to update our models iteratively as new data is acquired, and use interim model predictions to guide which experiments to prioritize to maximize predictive ability[79]. We will control for experimental conditions (assays on live viral isolates, full-genome or synthetic chimeric viruses, and the molecular backbone of the latter). Traits will be used as inputs to BNM's causal graph, to predict latent variables representing interconnected processes that contribute to SARSr-CoV QS infection in new hosts : receptor binding, cell entry, immune system interaction, and intracellular growth, all measured by our lab assays. These, in turn will **act as predictors for the ultimate outcomes of host pathogenesis and host jumping potential (Fig. 7)**. We will use published work on these genetic traits to put informative priors on strength and direction of interactions in the causal graph. We will use **prior-knowledge model simulations to select target sequences from our sampling for characterization and genome-sequencing**, to collect data that maximally enhances the predictive power of our model, and update these simulations iteratively throughout the experimental phase to continually guide QS selection. We will use regularizing priors to reduce over-fitting and select the most predictive variables in the final model.

Model validation using SARSr-CoV serology from previously-collected human samples and surveillance data. Active spillover of SARSr-CoV in our study region enables us to measure actual spillover risk to validate our models of QS jump potential. We will gather data on viral QS antibodies found in the local human population using LIPS assays on >2,000 previously-collected human sera (NIAID, Daszak PI) from people living close to our test cave sites in Yunnan Province, a sub-sample of which showed 2.7% seropositivity to bat SARSr-CoVs[13]. The IRB for

this work is current and covers proposed DEFUSE testing. We will design LIPS assays targeting high- and low-spillover risk SARSr-CoV QS, as done previously for SARSr-CoVs[13,80,81] and the novel SADS-CoV[14]. We will: **1)** insert different high- and low-risk SARSr-CoV N genes into pREN-2 vector (LIPS vector), first assessing N gene similarity to determination their potential cross-reactivity in a LIPS assay; **2)** determine LIPS assay specificity by producing polyclonal sera via injection of recombinant protein or attenuated virus into rabbits; **3)** validate LIPS assays by incubating antigens with their respective positive serum samples and the antigen antibody complex eluted using protein A/G beads; **4)** validate LIPS positive sera results by spike protein based LIPS and viral neutralization assay. As a confirmatory test, the positive samples from LIPS will be validated by virus neutralization assay. We will use these LIPS assays to test serum samples for presence of antibodies to high- and low-risk SARSr-CoV QS. We will validate predictions of jump potential and extend the BNMs to predict actual spillover probabilities by modeling bat-human contact rates with bats. We will use ecological data on bat hosts and human behavioral survey data collected previously from these individuals to estimate wildlife contact in predicting exposure measured by our LIPS assays.

Evolutionary modeling and simulation to predict potential strains. Our Bayesian network modeling will generate predictions of the spillover risk of QS sequences we identify. To examine risk associated with the total viral population, we will model and simulate evolutionary processes **to identify likely viral QS that our sampling has not captured, and viral QS likely to arise in the future ("QS_x").** We will use a large dataset of S protein sequences and full-length genomes generated from prior work and DEFUSE fieldwork to estimate SARSr-CoV substitution rate and its genome-wide variation using coalescent and molecular clock models within a Bayesian MCMC framework[82]. We will estimate SARSr-CoV recombination rates at the cave population level using these data and Bayesian inference[83,84]. We will apply RDP[85], similarity plots, and bootscan to identify recombination breakpoints and hotspots within the SARSr-CoV genome as done previously[34], now extended to the full genome. Using these estimates we will simulate the evolution of the SARSr-CoV QS virome using a forward-time approach implemented in simulators that model specific RNA virus functions (e.g. VIRAPOPS[86]). We will predict the rate at which new combinations of genetic traits can spread in viral populations and compare recombination rates among caves and bat communities. Our forward-simulated results **will provide a pool of likely unknown and future QS_x species.** Using these and our SEM model for spillover risk, **we will predict the QS_x most likely to arise *and* have spillover and pathogenic potential.** We will use evolutionary simulation results to iteratively improve our Bayesian network model. The number of genetic traits with potential for prediction of pathogenicity is large, so we will perform variable reduction using tree-based clustering, treating highly co-occurring traits as joint clusters for prediction. We will generate these clusters from all SARSr-COV sequences from DEFUSE fieldwork and prior work. As trait clusters may be modified through recombination, we will use our forward-evolutionary modeling to predict how well trait clusters will be conserved, retaining only those unlikely to arise in unknown or QS_x genomes. This will enable a trade-off between increased predictive power based on current samples and generalizability to future strains that have not yet evolved.

Technical Area 2

Immune modulation approach to reducing bat SARSr-CoV spillover risk. Our work shows that the following unique immunological features of bats may explain their capacity to harbor high viral loads with minimal clinical signs: a) bats maintain constitutively high expression of IFNα that may respond to and restrict viral infection[17]; b) several interferon activation pathways are dampened, e.g. STING (a central cytosolic DNA-sensor molecule to induce interferon) dependent and TLR7 dependent pathways[16]; c) the NLRP3 dependent inflammasome pathway is dampened, and key inflammation response genes like AIM2 are not present in bats[87,88]. These traits may be due to bat immune-sensing pathway adaptation as a fitness cost of flight[15]. We hypothesize that bat virus replication will likely be restricted quickly by constitutively expressed IFNα in bats, resulting in lower B/T cell stimulation due to lower viral stimuli. Second, dampened interferon and inflammasome responses will result in lower cytokine responses that are required to trigger T/B cell dependent adaptive immunity (e.g. antibody response), ultimately resulting in suppression of viral replication and shedding. **We and others have demonstrated proof-of-concept of this phenomenon:** Experimental Marburg virus infection of Egyptian fruits bats, a natural reservoir host, resulted in widespread tissue distribution with low viral load, brief viremia, low seroconversion and a low antibody titer that waned quickly, suggesting no long-term protection is established[89-91]. Poor neutralizing antibody responses occur after experimental infection of bats with Tacaribe virus[92] and in our studies of experimental infection of bats with SARS-CoV (Wang, unpubl.). We also successfully showed that bat interferon can inhibit bat SARSr-CoVs[21]. We hypothesize that use of immune modulators that upregulate the naturally low innate immunity of bats to their viruses, will transiently suppress viral replication and shedding, reducing the host jump risk. We further hypothesize that because *Rhinolophus* bats are long-lived (20+ yrs in the wild), most bats in a population will have been exposed to a range of SARSr-CoV QS at our sites. Specifically targeting upregulation of their adaptive immunity (immune memory) to high-risk viral strains may lead to heightened clearance of high-risk strains. We will evaluate two immune modulation approaches to defuse spillover of SARSr-CoVs from bats to humans: **1) Broadscale Immune Boosting strategies (Wang, Duke-NUS):** we will apply immune modulators like TLR-ligands, small molecule RIG-like receptor (RLR) agonists or bat interferon in live bats, to up-regulate their innate immunity and suppress viral replication and shedding; **2) Targeted Immune Boosting (Baric, UNC):** the broadscale immune boosting approach will be applied in the presence of chimeric immunogens to activate immune memory in adult bats and boost clearance of high-risk SARSr-CoVs. We will use novel chimeric polyvalent recombinant S proteins in microparticle encapsidated gels for oral delivery and/or virus adjuvanted immune boosting strategies where chimeric recombinant SARSr-CoV S are expressed by raccoon poxvirus. Both lines of work will begin in Year 1 and run parallel, be assessed competitively for efficiency, cost, and scalability, and successful candidates from captive animal trials will be used in live bat trials at our test cave in Yunnan. The finding of low innate immunity across bats suggest that immune boosting could be broadly applicable to bat genera and viral families.

Broadscale immune boosting (Duke-NUS). We will work on the following key leads to identify the most effective approach to up-regulate innate immunity and suppress viral loads. *Toll-like receptor (TLR)/RIG-I Like Receptor (RLR) ligands:* Our work indicates a robust response in live bats to TLR-stimuli like polyI:C as measured by transcriptomics on spleen tissue (**Fig. 8**), liver, lung and lymph node, with matched proteomics to characterize immune activation *in vivo*. These activation profiles will be used to assess bat immune response to different stimuli and identify those which lower viral load in our experimental system at Duke-NUS (below).



Fig. 8: Pathway analyses from Ingenuity Pathway Analysis (IPA) of whole spleen NGS after stimulation with either LPS or polyI:C. Z-score increase over control bats is indicated as per scale, and suggests strong activation of many pathways.

We will also stimulate the RIG-I pathway with 5'pppDSRNA, a mimetic of the natural RIG-I stimulant that will activate functional bat IFN production pathways, as shown in a mouse model that cleared SARS-CoV, IAV and HBV[18,19]. *Universal bat interferon:* We will design a conserved universal bat interferon protein sequence with artificial gene synthesis and produce recombinant protein by cleavable-affinity-tagged purification of supernatant from over-expressing bat cells, as used previously for recombinant *Pteropus alecto* IFNα[17,93] and CSF-1/IL-4. Utilization of a universal IFN for bats will overcome species-dependent response to the ligand, allowing the use of IFN throughout broad geographical and ecological environments and across many bat species. We have already produced recombinant non-universal, tagged, bat IFN that induce appropriate immune activation (**Fig. 9**). This ligand has been shown to reduce viral titers in humans, ferrets and mouse models intranasally and orally[18,19,94]. Interferon has been used clinically in humans as an effective countermeasure when antiviral drugs are unavailable, e.g. against filoviruses[20]. Interferon is known to be toxic, therefore we will carefully examine dose tolerance in bats and assess clinical effects of the treatment. We have shown that replication of SARSr-CoV is sensitive to IFN treatments[21]. The successful delivery, immune activation and outcome on the host will be characterized thoroughly to optimize rapid immune activation.

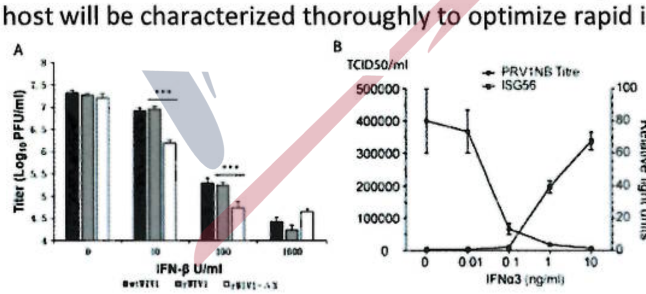


Fig. 9: Bat viruses are sensitive to IFN treatments. A) Recombinant bat SARS-related coronavirus WIV1 replication was inhibited by human IFN-β in a dose dependent manner in Vero cells. B) Bat reovirus PRV1NB replication was inhibited by recombinant bat IFNα3 in a dose dependent manner in bat PakiT03 cells.

Boosting bat IFN by blocking bat-specific IFN negative regulators: Uniquely, bat IFNα is naturally constitutively expressed but cannot be induced to a high level, indicating a negative regulatory factor in the bat interferon production pathway[17,95]. We will use a *Pteropus alecto* CRISPRi library pool that we have created covering multiple RNA targets in every gene in the *P. alecto* genome (Wang, unpubl.

data). Genes affecting influenza replication in bat cells have already been identified using this library. Using CRISPRi we will identify negative regulator genes and screen for compounds targeting them to boost the inducibility of the IFN system in a shorter time-frame. Based on previous work[96-98], it is highly likely this will be a conserved pathway across all bats. *Activating dampened bat-specific innate immune pathways which include DNA-STING-dependent and TLR-dependent pathways:* We have shown that mutant bat STING or reconstitution of AIM2 and functional NLRP3 homologs restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence. By identifying small molecules to directly activate pathways downstream of STING or TLR/RLRs, such as TBK1 activation, we will activate bat innate defense by interferons, promote viral clearance and, we hypothesize, significantly reduce viral load in bats. *Validation in a bat-mouse model.* Various CoVs show efficient infection and replication inside the human host but exhibit defective entry and replication using mouse as a host due in part to differences in DPP4 and ACE2 receptors.

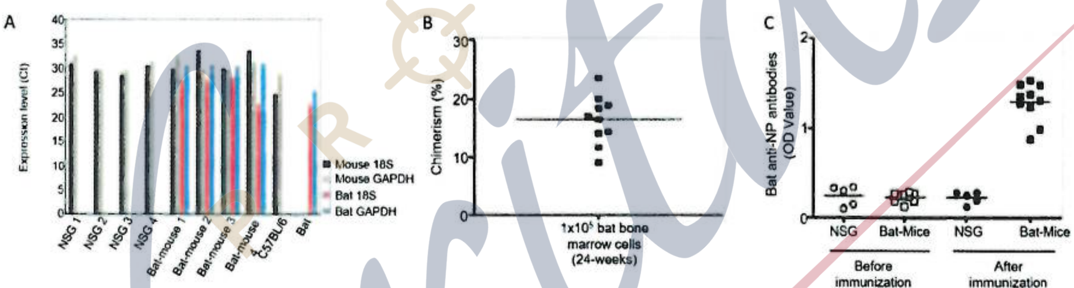


***Fig. 10**: A) Presence of at-specific qPCR in reconstituted mice after 12 weeks. B) chimeric ratio of bat-mouse cells in circulation after 24 weeks. C) Specific antibody response to a KLH-tetanus antigen generated by bat-reconstituted mice.*

We have shown efficient reconstitution of irradiated mice using bat bone marrow from multiple species, including *E. spelaea* (**Fig. 10**), including reconstitution of bat PBMC's in the mouse, presence of circulating bat cells and generation of bat-specific antibodies in mice incapable of producing an antibody response. **This 'batified' mouse model** can be utilized for both circulating infection of SARS-CoV (in the immune compartment only) and as a model for generating bat-specific antibodies against CoV proteins. Efficient validation of infection into bat cells will be used to validate the infectivity of the viruses and generation of bat antibodies will facilitate validation of the best proteins/peptide to elicit an effective immune response.

Targeted immune boosting (UNC). To boost targeted adaptive immunity (immune memory) in wild bats chronically exposed to circulating SARSr-CoV QS, we will inoculate with chimeric S glycoproteins in the presence of the broadscale immune boosting agonists above. We have developed novel group 2b SARSr-CoV chimeric S glycoproteins that encode neutralizing domains from phylogenetically distant strains (e.g. Urbani, HKU3, BtCoV 279, ~25% diversity). The chimeric S programs efficient expression when introduced in the HKU3 backbone full length genome, and elicits protective immunity against multiple group 2b strains. We will develop robust expression systems for SARSr-CoV chimeric S using ectopic expression *in vitro*. We will work with Dr. Ainslie (UNC-Pharmacy) who has developed **novel microparticle delivery systems and dry powders for aerosol release** that encapsidate recombinant proteins and adjuvants (innate immune agonists) that we will use for **parallel broadscale immune boosting strategies**

± chimeric immungens. Simultaneously, we will introduce chimeric and wildtype S in raccoon poxvirus (RCN), in collaboration with Dr. Rocke and confirm recombinant protein expression, first *in vitro* and in the Duke-NUS bat colony, prior to any field trial. The goal is to develop a suite of reagents to remotely reduce exposure risk in high risk environmental settings. *Chimeric SARSr-CoV S Immunogens:* CoVs evolve quickly by mutation and RNA recombination[34,99], and encode neutralizing epitopes in the amino terminal domain (NTD), RBD and S2 portion of the S glycoprotein[66,100,101], providing a strategy to build chimeric immunogens that induce broadly cross reactive neutralizing antibodies. Given the breadth of SARSr-CoV circulating in natural settings, chimeric immunogens will be designed to increase the breadth of neutralizing epitopes across the group 2b phylogenetic subgroup[48]. Using synthetic genomes and structure guided design, we fused the NTD of HKU3 with the SARS-CoV RBD with the remaining BtCoV 279/04 S glycoprotein molecule, introduced the chimeric S glycoprotein gene into the HKU3 genome backbone (25% different than SARS-CoV, clade 2 virus) and recovered viable viruses (HKU3-S_{mix}) that could replicate in Vero cells. We inserted HKU3$_{mix}$ S glycoprotein gene into VEE virus replicon vectors (VRP-$S_{chimera}$) and demonstrated that VRP vaccines protect against lethal SARS-CoV challenge and virus growth. VRP-S_{HKU3} and VRP-S_{279} both protect against HKU3$_{mix}$ challenge and growth *in vivo*, demonstrating that neutralizing epitopes in the HKU3$_{mix}$ S glycoprotein provide broad cross protection against multiple SARSr-CoV strains. In addition to using these immunogens as a targeted broad-based boosting strategy in bats, we will produce other chimeras for more focused immune targeting of known high risk strains. We will use the Protein Expression Core at UNC (https://www.med.unc.edu/csb/pep) to produce codon optimized, stabilized and purified prefusion SARS-CoV glycoprotein ectodomains[23]. Purified recombinant protein will be used for inclusion in delivery matrices (e.g. purified powders, dextran beads, gels – see below) with broadscale immune agonists (adjuvants, Duke-NUS). *2nd Generation Chimeric S glycoprotein Immunogen Design and Testing:* We will produce a chimeric SHC014 NTD/SARS-CoV-RBD/HKU3 S C terminal recombinant S immunogen (HKU3-S_{S014}), for more focused immune targeting on known high and low risk strains designated from our experimental and modeling analyses. The recombinant HKU3-S_{S014} S genes will be sent to Dr. Rocke for insertion into the raccoon poxvirus vaccine vector. Using established techniques, we will characterize S expression and provide virus vectors to Prof. Wang for immune boosting trials at Duke-NUS, and if successful in the field (Prof. Shi). The human codon optimized HKU3-S_{S014}, and HKU3-S_{mix} recombinant chimeric spike glycoproteins will be expressed and purified by the UNC proteomics core, producing mg quantities for inclusion in nanoparticle and microparticle carriers in collaboration with Dr. Ainslie. We will produce WIV-S_{S014} and HKU3-S_{mix} glycoprotein expression will be validated by Western blot and by vaccination of mice, allowing us to determine if the recombinant protein elicits neutralizing antibodies that protect against lethal SARS-CoV and SHC014 challenge. We will produce enough material for *in vivo* testing in mice and in bats. We will validate recombinant virus glycoprotein expression by Western blot and by vaccination of mice, to determine if the recombinant protein elicits neutralizing antibodies that protect against lethal SARS-CoV, HKU3-Smix and SHC014 challenge. We will survey the RNAseq data for evidence of complex S glycoprotein gene RNA recombinants in the SARSr-CoV population genetic structure. If present, we will synthesize 2-3 potentially effective recombinant S genes, insert these genes into SHCO14 or HKU3 genome backbones and VRP, and characterize their

viability and replicative properties in cell culture and in mice. We will produce immunogens and evaluate their ability to protect against infection.

Adjuvant and Immunogen Delivery Vehicles. Dr. Ainslie (UNC) has developed the biodegradable polymer acetalated dextran (Ac-DEX) for the delivery of antigens and adjuvants in vaccine applications. Ac-DEX has advantages over other polymers for vaccine development: it is easily synthesized and scalable using an FDA-approved one-step method to move from water-soluble to insoluble[102-104]; it is acid sensitive which improves antigen presentation; microparticles (MPs) are small (5-8µm) so can be phagocytosed by DCs and traffic to the lymph node for efficient molecule delivery[105]; MPs are pH-neutral, safe[106], stable outside the cold-chain[107], can be aerosolized or delivered in sprays or gels[102,108]; and we have previously encapsulated Poly (I:C)(1), resiquimod[105], and a STING agonist into our novel MPs[109], providing proof-of-concept that this significantly enhances the activity of the TLR agonist. We have displayed better efficacy than state-of-the-art FDA-approved inactivated flu virus (Fluarix) in a ferret model of influenza[110], using HA with encapsulated STING cyclic [G(3',5')pA(3',5')p][111]. *Microparticle Performance Metrics in vitro and in Rodents and Bats:* We will encapsulate Poly (I:C), resiquimod (TLR 7) or other innate immune agonists to enhance type I interferon production in in consultation with Prof. Wang. Agonist laden particles will be made separately or in combination with recombinant SARS-CoV chimeric spike proteins, encapsulated into our aerodynamic MPs and nanoparticles. *Viral infection models in* Eonycteris *spp. (Duke-NUS) and wild-caught* Rhinolophus *spp. (Wuhan Inst. Virol.) bats:* To test and compare the efficacy of the immune modulating approaches above, we will use our cave-nectar bat (*Eonycteris spelaea*) breeding colony infected with Melaka virus (family *Reoviridae*) which infects this species[112,113]. First, we will take wing punch biopsies from 3 individuals to sequence their ACE2 receptor gene. This will be inserted into human cell lines to pre-screen viral strains for binding. Those that bind will be used for *in vivo* expts. We will use two coronaviruses (SARSr-CoV WIV1 and MERS-CoV) in ABSL3. SARS and MERS infection studies are already underway in *Eonycteris* and *Pteropus* cell lines and primary immune cells. Our *E. spelaea* colony has now reached a sustainable population for infection experiments and the ABSL3 facility has been outfitted with bat-specific cages. The planned pilot *in vivo* infection of *Eonycteris* bats with Melaka Virus and MERS will be completed by July 2018. Previous infection studies were completed in *Pteropus* and *Rhinolophus* bats in Australia by L-F Wang at CSIRO, AAHL and an additional *Pteropus* infection trial is currently planned through the University of Queensland in Australia. At WIV, 20 adult wild *Rhinolophus* spp. bats (10 of each sex) will be captured at our test cave site, housed within ABSL3, ACE2 receptor genes sequenced and used to pre-screen spikes as above, then bats will be tested using PCR and serology for current and prior exposure to SARSr-CoVs, and inoculated with WIV1, WIV16 or SHC014. For all experiments, viral loads will be measured by qPCR, titration of produced virus, NGS transcriptomics and viral-specific nanostring probes added to the immunoprofiling panel. Antibody responses will be measured by LIPS assay, as described previously. In addition to direct *in vivo* delivery of ligands, aerosolized and liquid-phase deployment methods suitable for a cave-like environment will be tested, in collaboration with UNC, NWHC and PARC. This approach allows us to test our immune-boosting strategies, in a safe and controlled environment, prior to expanding to field-based evaluation. The experimental protocols and analytical methods used for the *E. spelaea* colony, with a focus on

internal-normalization and small amounts of sample materials (including nanostring analysis from whole blood-droplets), will be replicated to analyze experimental infection of wild-caught *Rhinolophus* spp. bats at WIV and in the test cave trail in TA2.

Delivery system development (NWHC). We have previously developed, safety- and efficacy-tested, and registered oral vaccines and delivery methods to manage disease in free-ranging wildlife including a plague vaccine for prairie dogs[30], a rabies vaccine for bats[31] and strategies for white-nose syndrome (unpubl. data)[114]. As previously shown for rabies vaccine in bats, we will trial sticky edible gels that bats groom among each other to deliver immune modulators and recombinant SARSr-CoV spike proteins to *Rhinolophus* bats, including trials of them combined with poxvirus vectors and nanoparticles/nanoemulsions that enhance uptake through transdermally. Poxviruses are effective viral vectors for delivering vaccines to wildlife [30,115,116], and can replicate safely at high levels in bats after oronasal administration[32]. We have demonstrated proof-of-concept and safety in bats with modified vaccinia Ankara (MVA) and raccoon poxvirus (RCN) vectors using *in vivo* biophotonic imaging[31]. RCN replicated to higher levels in bats than MVA, even via the oral route, and was found to be safe. We used raccoon poxvirus-vectored novel rabies glycoprotein (mosaic or MoG) and demonstrated protective efficacy in bats after oronasal and topical administration[31] (**Fig. 11**).

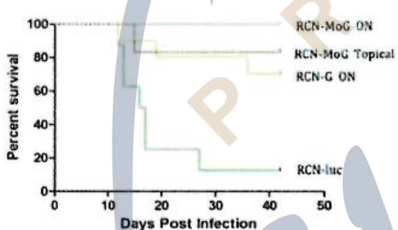


***Fig. 11**: Vaccine efficacy, rabies challenge in Epstesicus fuscus immunized with raccoon poxvirus expressing a mosaic G protein (RCN-MoG) oronasally (ON) or topically in comparison to RCN expressing typical G protein or luciferase (negative control).*

Poxviruses are safe in a wide variety of wild and domestic animals, and allow for large inserts of foreign DNA. We have previously used a raccoon poxvirus vectored vaccine expressing plague antigens to manage plague caused by *Yersinia pestis* in prairie dogs. We incorporated the biomarker Rhodamine B (RB) into baits to assess uptake by target and non-target species [114,117] (**Fig. 12**). RB is visible under a UV microscope until the hair grows out (~50 days in prairie dogs). We have since conducted a large field trial that demonstrated vaccine efficacy in four species of prairie dogs in seven western states[30].



Fig. 12: Prairie dog hair and whisker samples under fluorescence microscope to determine uptake of baits containing Rhodamine B. a) 20 days after bait distribution, b) 16 days after bait distribution, c) and d) controls (note natural dull fluorescence).

Transcutaneous delivery: To trial a strategy that avoids use of live agents, we will use nanoparticles to increase transcutaneous delivery efficiency[118]. We will use poly lactic-co-glycolic acid (PLGA) to encapsulate immune modulators as a method of transcutaneous delivery of vaccine to bats via dendritic cell uptake[119], as has been shown for delivery of TLR agonists and antigens simultaneously to mice[120]. This approach will be competitively trialed against ac-DEX, with and without adjuvants[121] which enhance the immune response in mice to SARS-CoV spike proteins[24]. Initial trials will be conducted in the USA with locally acquired insectivorous big brown bats (*Eptesicus fuscus*) which we have maintained and housed for several experiments previously[31,32]. We will treat bats via topical application with various test formulations that include the biomarker Rhodamine B (RB), co-

house them with untreated bats, and monitor transfer between bats by collecting hair and whiskers for biomarker analysis. *Initial field trials:* High rates of grooming within bat colonies allow effective transfer or products among a colony. In biomarker trials in Peru, RB-labeled glycerin jelly yielded a rate of transfer from 1.3 – 2.8 bats for every bat marked. We will conduct initial trials with each of the delivery vehicles in local US insectivorous bats in their natural setting. Within one week of application of varying doses, bats will be trapped at the cave entrance using mist nets or Harp traps and hair will be collected to assess the rate of uptake via biomarker analysis, then released. After we have determined optimal approaches for mass delivery, we will test them on wild-caught captive *Rhinolophus* bats (WIV), then in our three cave sites in Yunnan Province. Biomarker will be used to assess rates of uptake (and non-target species contamination) and these data used in modeling studies to help determine the optimal rates of application of immunomodulating agents. *Innovative Aerosol Approach to Bat Inoculation:* Once we have confirmed uptake in laboratory studies, we will assess scalable delivery methods in local caves and hibernacula using biomarker-labeled mediums without immunomodulatory substances. In collaboration with Dr. Jerome Unidad of Palo Alto Research Center (PARC), we will use an innovative aerosol platform technology unique to PARC to design a field-deployable prototype for use in cave settings. The Filament Extension Atomization (FEA)[122] technology can spray fluids with a wide-range of viscosities ranging from 1mPa-s (the viscosity of saliva and most aqueous vaccine formulations) up to 600Pa-s (the viscosity of creams and gels for topical delivery) using a roll-to-roll misting process (https://www.parc.com/services/focus-area/amds/) that results in narrowly-dispersed droplets with tunable sizes from 5-500 microns. FEA technology is compatible with all the formulations of interest to project DEFUSE, including aqueous formulations intended for conventional spraying and the edible gels and creams for topical delivery, with no limit on bioactive ingredient loading. FEA can be a universal delivery platform for direct spraying onto bats with the formulation geared towards bio-efficacy. Potential form factors for a prototype cave-based spray system are shown in **Fig. 13**.

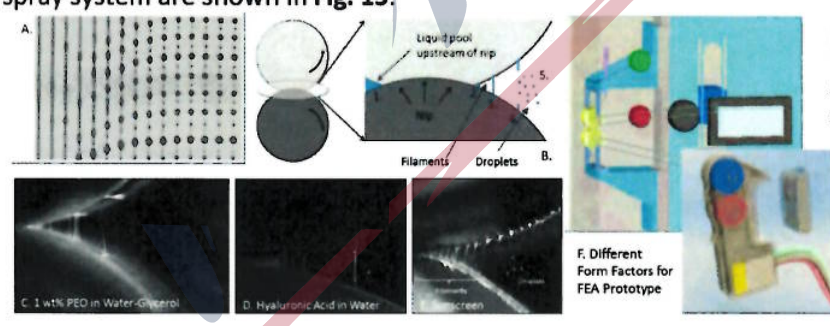


***Fig. 13**: PARC FEA Technology – A. Beads-on-a-string structures in viscoelastic fluids[123], B. Parallelization of filament formation and droplet break-up in an FEA roller system, C.-E. Images from high speed videos of representative fluids sprayed with FEA, F. Potential field-deployable prototype for Project DEFUSE (benchtop, handheld).*

PARC and NWHC will conduct initial prototype trials on US cave bats. PARC will then develop the prototype to a form that will be used for the proof-of-concept demonstration at the test sites in the Kunming bat caves, Yunnan, China. The field-deployable system will be motion-actuated, and on a timer so that bats will be targeted at fly-in and fly-out to avoid non-target species (e.g. cave swiftlets).

Dynamic circulation modeling to optimize deployment strategy. To select immune boosting,

and multiple delivery options and schedules, we will simulate deployment using a model of viral circulation in cave bat populations. The model will be fit to data from our three-cave test system but designed to be robust and generalizable to other cases. We will simulate outcomes under a variety of different deployment scenarios to produce conservative estimates of optimal application under real-world conditions. *Fit stochastic viral circulation models to longitudinal sampling data:* We will use longitudinal viral prevalence, mark-recapture data, telemetry and infrared camera data collected during our field sampling to parameterize and construct models of bat population dynamics and viral circulation in our test caves.

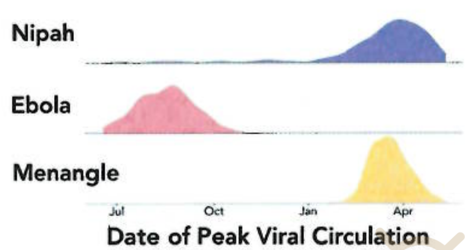


Fig. 14*: Modeled seasonal circulation of viruses modeled from longitudinal sampling of viruses in bat colonies in Bangladesh.*

We will modify existing models that extract temporal dynamics developed for multiviral systems based on longitudinal studies in Bangladesh[124] **(Fig. 14)**. We will use a simple but robust stochastic SIR process model with immigration and emigration and flexible, nonlinear contact rates between bats[125] to capture a wide range of viral dynamics from intermittent viral outbreaks to regular, endemic circulation with a relatively small number of parameters. We will fit these models to our sampling data using the **partially observable markov process (pomp)** framework[126], allowing estimates of the underlying latent dynamic disease transmission process, accounting for and separating natural stochasticity of viral circulation and observation error in sampling. We will validate our models via temporal cross-validation and by testing the results of a fit from two cave sites on data from a third. *Simulate circulation under a set of plausible deployment scenarios.* Using the top performing sets of immune boosting molecules from captive trials, and the most effective delivery media and methods from cave studies, we will use the stochastic SIR model to generate simulations of viral circulation under a series of deployment scenarios in our test caves. These scenarios will cover a range of plausible intensities, frequencies, and combinations of suppression strategies and will incorporate uncertainty in the efficacy of each treatment strategy. From these simulations, we will estimate the expected degree and time period of suppression of viral circulation and shedding and determine the optimal scenario for deployment in our focal study caves. *Test robustness of deployment strategies under broader conditions:* We anticipate deployment is likely to occur under highly varied species population and compositions, with uncertain estimates based on rough observations, with varied uptake and efficacy of treatment due to different environmental conditions, and with limited time and resources. We will simulate deployment under many potential conditions to determine optimal deployment under each, and strategies that are conservative and robust to uncertainties and limitations.

Proof-of-concept deployment of immune modulation molecules in test caves in Yunnan Province, China. We will deploy the most successful immune modulation molecules at our three test-caves in Yunnan Province, China. We will identify the primary entry/exit points and traffic patterns for each cavern used by bats during flyout by LiDAR mapping and infrared video surveillance, and position two PARC spray nozzles around the top (10 and 2 o'clock position, pointing down) of the primary opening. Depending on the size of the cave opening, we will

mount the nozzles on extendable booms to position them at the top of the cave mouth. Spray will be activated by bat movement to spray continuously or in a staggered manner during flyout and flyin to create an aqueous curtain that bats will fly through. We will determine the optimal duration of spraying using stochastic viral circulation models and real-time population size data. We assume a rate of glycerin gel transfer of 2.0, through grooming activity from preliminary data. Initial trials will use RB to assess coverage. Then, at the test cave, immune-modulation biologicals and biomarkers will be deployed, with biomarker only sprayed at two other control caves. The 4 months pre-deployment sampling will be followed by 4 months post deployment surveillance (**see sampling strategy**) commencing the day following deployment through which we will assess 1) nature and duration of individual bat immune response to the treatment and 2) population level SARSr-CoV viral shedding rates in the test and control caves based on fecal pellet screening. We will also screen for the biomarker to assess coverage achieved. This approach can be **scaled up** for a larger network of bat roosts by using our app to model bat roost connectivity (mixing patterns among local caves based on our recapture and telemetry data) and identify caves with the highest connectivity so that bats transfer the biological treatment to other caves at a rate that will dampen viral shedding over a much larger area.

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs). **Subcontracts: #1** to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting

treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the

problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots[49,127], estimates of unknown viral diversity[50], predictive models of virus-host relationships[7], and evidence of the bat origin of SARS-CoV[2] and other emerging viruses [128-131].

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential "pre-epidemic" risk associated with zoonotic virus cross species transmission[10-12,29,57,64].

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others[2,14,60,80,124] and genetic work linking bat immunology, flight, and viral tolerance[15,16,75,93]. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore Nat. Res. Foundation grant "Learning from bats" ($9.7M SGD).

Prof. Zhengli Shi is director of the Center for Emerging Infectious Diseases of the Wuhan Institute of Virology, Chinese Academy of Sciences and BSL3 and BSL4 lead. Her research focuses on traditional and high-throughput sequencing techniques for viral pathogen discovery. Since 2004, she has studied bat-borne viruses, leading the SARSr-CoV group discovery[2,3,34,67].

Dr. Tonie Rocke is a research scientist at the USGS National Wildlife Health Center. Her research focuses on the ecology and management of diseases in wild mammals (e.g. plague, monkeypox, rabies and white-nose syndrome). She leads a large-scale field trial of oral plague vaccination of wild praire dogs in the western U.S. [30-32,114,117].

Dr. Jerome Unidad is a Member of Research Staff at the Hardware Systems Laboratory at PARC. His research focuses on novel fluid delivery systems including aerosol delivery for high viscosity fluids, polymers and biomacromolecules. He is the technical lead in developing the FEA spray technology for consumer and biomedical applications, and additive manufacturing.

Section II CAPABILITIES

EcoHealth Alliance (EHA) is an international non-profit researching emerging zoonoses in >20 countries Asia, Africa and South America. EHA has pioneered modeling and analyses of the origins and drivers of emerging diseases, of the bat origins of emerging viruses, and the

dynamics of SARSr-CoVs, henipaviruses and other high-profile emerging pathogens. EHA is major consortium partner in the USAID-EPT-PREDICT program that has tested over 35,000 animals and discovered 200 new viruses to date.

University of North Carolina Medical School (UNC). The Baric Laboratory in University of North Carolina at Chapel Hill comprise biosafety level two facilities equipped to perform basic virology, immunology, and molecular biology as well as university space for breeding mice for the proposed studies. The Baric BSL-3 laboratories are approved and have the required equipment to perform all of the chimeric virus recovery and characterization and ventilated rodent caging to examine the bat coronaviruses within this proposal.

The National Wildlife Center (NWHC) contains specialized research laboratories and support areas, staff offices, and BSL-3 biocontainment animal research areas. Two, fully equipped laboratories in the research building are available at all times for the proposed work. NWHC's BSL-3 biocontainment animal research area is equipped with pathology incineration, steam sterilization equipment, and an ultraviolet radiation chamber so that all materials can be treated before leaving the biological containment area, and is maintained under negative air pressure. Trained animal care staff and a Veterinary Medical Officer are available to maintain, monitor and handle animals. The NWHC is fully equipped for animal medical procedures.

Palo Alto Research Center (PARC) is composed of 140+ professional full time researchers from physics, materials science, chemistry, biology, engineering, computer science, controls and ethnography fields. Facilities comprise two general purpose labs for conducting spray experiments and fluid mechanics measurements, a cell culture lab, chemistry labs, laser patterning and 3D printing facilities, a professional staffed model shop and electronics labs.

Wuhan Institute of Virology: includes BSL2, BSL-3, and BSL-4 laboratories, animal feeding rooms and other supporting facilities. The Biosafety Laboratory will carry out CoV research, sample testing, sequencing, binding assays, *in vitro* and *in vivo* work.

Duke-NUS Medical School; Singapore: The Duke NUS Animal BSL-3 facility is co-located with the SingHealth Animal Husbandry and Hospital in Northern Singapore. The Facility is a state-of-the-art modular laboratory equipped to safely carry out infectious diseases research. The Animal BSL-3 lab is equipped with virology, basic immunology, and molecular biology capacity, and is equipped for handling caged animals including rodents, nonhuman primates and bats.

Intellectual property rights: PARC asserts restrictions on the following noncommercial item: An Efficient Method for Collecting Droplets of Strain Hardening Viscoelastic Fluids in a Spray Device, to be used in device development towards Project DEFUSE. PARC will develop the technology exclusively at private expense, and asserts that the USG has limited rights on technical data associated with the device. PARC is willing to negotiate in good faith with the government or technology transition partner for relevant background intellectual property to support transition. There are no restrictions on Commercial property rights.

Related research:

Daszak, PI on subcontract, USAID-EPT1&2, PREDICT: Consortium partner lead, Exec. Board member, Modeling & Analytics lead for two 5-year contracts ($75 million; $138 million) to conduct surveillance for novel viruses in wildlife in >25 countries globally, capacity build, assess behavioral risk, and manage databases. EHA subcontracts >$35 million. >1000 viruses discovered, 10,000 samples collected. Papers published in *Science, Nature, Lancet.*

Daszak, PI, NIAID: Understanding the Risk of Bat Coronavirus Emergence: 5-year grant to research spillover risk of novel SARSr-CoVs in China. $2.8 million, 5 subawards. Published work in *Science*, *Nature*, *PNAS*.
Daszak, Chief-of-Party, USAID: Infectious Disease Emergence & Economics of Altered Landscapes: PI on 3-year $2.5 million contract to analyze economics of land use change and disease emergence in Malaysia.

Section II STATEMENT OF WORK

PHASE 1

PI-TA-01 Task 1: Conduct longitudinal bat sampling and ecological data collection from field sites in Southern China to obtain data for experimental studies and modeling (EHA)
Sub-Task 1.1 Apply for and obtain IACUC and ACURO approval and appropriate permits in China for bat sample collection and field intervention pilot (EHA). ***Sub-Task 1.2*** Collect monthly specimens from bats at cave sites in Yunnan, China for SARSr-CoV screening and sequencing. Oral, fecal, and blood sample collected from 360 *Rhinolophus spp*. bats per month using live-capture and non-invasive sampling. Specimens shipped to laboratory for analysis. Associated morphological, demographic, and physiological data for individual bats collected (EHA, consultant Zhu). ***Sub-Task 1.3*** PIT tagging to assess bat connectivity and roost fidelity. All sampled bats marked with Passive Integrated Transponder tags. Radio frequency identification data loggers installed at each cave entrance for remote capture-recapture monitoring. (EHA, consultant Zhu). ***Sub-Task 1.4*** Satellite telemetry to assess bat home range size and connectivity. Mark 36 *Rhinolophus sp*. bats with 1g ICARUS satellite tags. (EHA, consultant Zhu). ***Sub-Task 1.5*** Real-time monitoring of bat populations. Conduct LiDAR cave surveys and establish remote IR thermal cameras at roost entrances for population size monitoring. Optimize algorithms for image recognition. (EHA, consultant Zhu). ***Sub-Task 1.6*** Develop and maintain project-wide database. Secure, cloud-hosted database will store all data collected and generated from field, lab, and experimental work, including code and generated data from modeling. (EHA)
Milestone(s): 1.1 Animal care and use approval and wildlife sampling permits obtained; 1.2 monthly collection of bat specimens and associate host data completed; 1.3 dataloggers and 1.5 IR cameras installed; 1.4 bat transmitters launched and data collection successful; 1.6 database built and tested; 1.6 field data entered into database monthly.
Deliverables: Specimens from 3,240 bats and fecal pellets collected from high-risk reservoir populations which have been obtained with all proper permits and permissions and shipped to WIV for analysis; real-time telemetry and mark-recapture data uploaded and made available to DARPA collaborators; completed database maintained.

PI-TA-01 Task 2: Construct models to predict bat species distributions and locations of greatest viral spillover risk (EHA).
Sub-Task 2.1 Construct joint species distribution models to predict bat community in caves across S. and SE Asia and identify high-risk geographic hotspots for viral spillover (EHA). ***Sub-Task 2.2*** Machine learning models using host and ecological traits to predict presence of viruses with zoonotic potential in bats (EHA) ***Sub-Task 2.3*** Non-parametric viral richness estimators to predict as-of-yet unsampled viral diversity. ***Sub-Task 2.4*** Develop prototype 'spatial viral spillover risk' app for the warfighter (EHA)

Milestone(s): 2.1 Joint species distribution model fit for Asian Bats, Cave-level predictions of bat community composition and viral diversity, 2.2 and 2.3 predictions of viral diversity and jump potential per bat spp. Prediction validation for 2.1-2.3; 2.4 Prototype app produce for β-testing, prototype app successfully field tested.
Deliverables: Deployable models of bat community composition and per-species viral diversity and zoonotic jump potential. Development of fully functional and user-friendly application.

PI-TA-01 Task 3: Screen, characterize and isolate SARSr-CoV QS_0 from bat samples (WIV)
Subtask 3.1 PCR screening of longitudinal specimens from target bat species (WIV). ***Subtask 3.2*** Genetically sequence SARSr-CoV spike proteins from PCR-positive samples (WIV). ***Subtask 3.3*** Develop and recover recombinant viruses with spike proteins from novel SARSr-CoVs (Duke-NUS). ***Subtask 3.4*** Identify the presence of low abundant, high risk SARSr-CoV, based on deep sequencing data (UNC)
Milestone(s): CoV prevalence and genetic diversity quantified; full genomes recovered
Deliverables: Library of PCR-positive specimens. Full sequencing of spike proteins. Creation of recombinant viruses to be used in task 4. List of potential higher risk SARSr-CoV QS.

PI-TA-01 Task 4: Experimental assays of SARSr-CoV QS jump potential (UNC)
Sub-Task 4.1 Conduct pre-screening via structural protein modeling, mutation identification, pseudovirus assays. (UNC). ***Subtask 4.2*** Conduct *in vitro* testing of chimeric viruses against host cell lines (UNC). ***Subtask 4.3*** Assess *in vivo* pathogenesis in hACE2 transgenic mice (UNC). ***Subtask 4.4*** Validate results from chimeric viruses with full-genome QS (UNC). ***Subtask 4.5*** Test synthetic modifications to viral spike proteins including RBD deletions, S2 Proteolytic Cleavage and Glycosylation Sites, N-linked glycosylation (UNC). ***Subtask 4.6*** Test effects of low-abundance, high-consequence micro-variations on jump potential. (UNC)
Milestone(s): Initiation and completion of each experimental sub-task.
Deliverables: Laboratory confirmed list of higher risk SARSr-CoV QS with zoonotic capability. Candidate SARSr-CoV for animal experiments. Data made available.

PI-TA-01 Task 5: Build and test Bayesian network models to predict genotype-phenotype spillover potential of high-risk SARSr-CoV strains. (EHA).
Subtask 5.1 Make predictions using prior data to guide QS selection for characterization (EHA).
Subtask 5.2 Update model predictions based on real-time data from viral *in vitro* and *in vivo* testing (EHA).
Milestone(s): Completion of preliminary model using prior data, tested and refined model using real-time data from the project.
Deliverables: Source code and model outputs from functional Bayesian predictive model for the risk of spillover of high-risk SARSr-CoV strains.

PI-TA-01 Task 6: Validate model predictions using SARSr-CoV serology from previously-collected human samples and surveillance data (EHA, WIV, Duke-NUS).
Subtask 6.1 Design Luciferase immunoprecipitation system (LIPS) assays to high- and low- jump risk SARSr-CoV QS_0 we have characterized (WIV). ***Subtask 6.2*** Determine specificity of LIPS assays by recombinant protein or attenuated virus inoculation into rabbits (WIV). ***Subtask 6.3*** Validate LIPS assays using positive serum samples, spike protein based LIPS and viral neutralization. (WIV). ***Subtask 6.4*** Test previously-collected human sera from Yunnan Province

to assess SARSr-CoV QS spillover (WIV). ***Subtask 6.5*** Validate BNM predictions of QS_0 jump potential and identify actual spillover probabilities using bat-human contact data (EHA).
Milestone(s): LIPS assays developed and validated; sera screened; Bayesian model validated
Deliverables: Data from serological assay validation and testing; New LIPS serology assays for specific SARSr-CoV QSs; Source code for validated model based on spillover evidence.

PI-TA-02 Task 7: Experimental testing of 'Broadscale Immune Boosting' using batified mice and captive bat colonies (Duke-NUS)
Subtask 7.1 Boost bat interferon (IFN) by blocking bat-specific IFN negative regulators (Duke-NUS). ***Subtask 7.2*** Activate dampened bat-specific innate immune pathways including DNA-STING-dependent and TLR-dependent pathways (Duke-NUS). ***Subtask 7.3*** Validate broadscale immune boosting in a bat-mouse model (Duke-NUS). ***Subtask 7.4*** Test immune modulation in captive *Eonycteris* sp. colony, using Malaka virus and SARSr-CoV infections. (Duke-NUS). ***Subtask 7.5*** Test targeted immune boosting in wild-caught captive *Rhinolophus* spp. (WIV)
Milestone(s): Initiation and completion of each experimental sub-task.
Deliverables: Experimental data; whole animal profiling of immune stimulants and associated response kinetics. Selection of one primary and two secondary ligands for use in subsequent viral challenge studies. Demonstrated animal models for broadscale immune boosting.

PI-TA-02 Task 8: Experimental testing of 'targeted immune boosting' using humanized mice and experimental bat colonies (UNC, NWHC, Duke-NUS, WIV)
Subtask 8.1 Develop chimeric SARSr-CoV S immunogens (UNC) ***Subtask 8.2*** Design and test 2nd generation chimeric S glycoprotein immunogens in humanized mice (UNC). ***Subtask 8.3*** Create raccoon poxvirus-vectored targeted immune boosting approach to be tested in captive bats at Duke-NUS (NWHC). ***Subtask 8.4*** Test targeted immune boosting in captive *Eonycteris* sp. colony, using Malaka virus and SARSr-CoV infections (Duke-NUS). ***Subtask 8.5*** Test targeted immune boosting in wild-caught captive *Rhinolophus* spp. (WIV)
Milestone(s): Initiation and completion of each subtask.
Deliverables: Chimeric SARSr-CoV S immunogens and poxvirus-vectored immune boosting molecules available for use. Proof-of-concept for targeted immune boosting approach in humanized mice and captive bats.

PI-TA-02 Task 9: Develop and assess transcutaneous delivery methods for immune boosting molecules (UNC, Duke-NUS, NWHC)
Subtask 9.1 Synthesize polymer acetalated dextran (Ac-DEX) microparticles (MPs) containing candidate broadscale and targeted immune boosting molecules (UNC, Duke-NUS). ***Subtask 9.2*** Test MP metrics *in vitro* and in rodents (UNC). ***Subtask 9.3*** Test MP safety in bats in Wisconsin and in Singapore (NWHC, Duke-NUS).
Milestone(s): Initiation and completion of each subtask.
Deliverables: Ac-DEX MPs that contain broadscale or targeted immune boosting molecules available for use. Data from MP efficacy and safety trials.

PI-TA-02 Task 10: Develop and assess delivery systems to deploy immune boosting molecules (NWHC, PARC)
Subtask 10.1 Test transcutaneous delivery methods using the biomarker Rhodamine (RB) on captive US bats (NWHC). ***Subtask 10.2*** Conduct field trials of RB-marked delivery substances on

wild US bats (NWHC). ***Subtask 10.3*** Develop prototype filament extension atomization (FEA) device (PARC). ***Subtask 10.4*** Trial FEA device using RB on US captive bats (NWHC)
Milestone(s): Initiation and completion of each subtask.
Deliverables: Data from transcutaneous delivery experiments in captive and wild bats. Prototype of FEA device. Proof-of-concept of FEA device delivery system.

PHASE II:
PII-TA-01 Task 1 (continued from PI-TA-01 Task 2): Updated 'spatial viral spillover risk' app based on laboratory and field experiments (EHA).
Subtask 1.1 Incorporate information on bat species risk from laboratory and field results (EHA). ***Subtask 1.2*** Incorporate risk-ranking algorithmns using geolocation features and host-pathogen characteristics (EHA). *Subtask 1.3* Link host species with viral diversity data from the project and previous data (EHA).
Milestone(s): Initiation and completion of each subtask.
Deliverables: Working prototype app that displays information by pathogen ranking, bat species ranking, and geographical ranking.

PII-TA-01 Task 2 (continued from PI-TA-01 Task 5): Build and test Bayesian network models to predict genotype-phenotype spillover potential of high-risk SARSr-CoV strains. (EHA).
Subtask 2.1 Estimate intra- and inter-species mutation and recombination rates in SARSr-CoV population (EHA). ***Subtask 2.2*** Simulate forward evolution to predict future and unsampled QS (EHA). ***Subtask 2.3*** Make predictions of likely future high-risk QS spillover (EHA)
Milestone(s): Initiation and completion of each subtask.
Deliverables: Source code and model outputs from functional model. Prediction of future QS variants. Identification of high-risk SARSr-CoV QS by network machine-learning model.

PII-TA-02 Task 3 (continued from PI-TA-02-10): Develop and assess delivery systems to deploy immune boosting molecules (NWHC, PARC)
Subtask 3.1 Design and optimize motion- and time- actuated facility for FEA prototype (PARC). ***Subtask 3.2*** Conduct field trials of RB-marked delivery substances using FEA motion-actuated prototype on wild bats in Wisconsin (PARC, NWHC).
Milestone(s): FEA deployment prototype designed; field trials completed.
Deliverables: Optimized FEA prototype with motion- and time- actuated facility. Proof-of-concept FEA delivery of RB-marked substances in wild bats.

PII-TA-02 Task 4: Build and test dynamic circulation models to optimize deployment strategy
Subtask 4.1 Develop robust stochastic SIR process model with immigration/emigration and flexible nonlinear contact rates among bats (EHA). ***Subtask 4.2*** Fit SIR model to sampling data from Yunnan test cave using partially observable markov process framework and validate via temporal cross-validation (EHA). ***Subtask 4.3*** Simulate viral circulation under series of plausible deployment scenarios to determine optimal scenario for deployment at test cave sites (EHA). ***Subtask 4.4*** Test robustness of deployment strategies under broader conditions.
Milestone(s): Initiation and completion of each subtask.
Deliverables: Source code and outputs from dynamic circulation models; Optimized scenario for deployment.

PII-TA-02 Task 5: Demonstrate accuracy of risk/pre-emption models then deploy most effective molecule delivery methods to suppress viral shedding in multispecies bat colonies of Yunnan Province caves (EHA, PARC, NWHC, Duke-NUS, UNC)
Subtask 5.1 Identify specific sites (entry, exit points), identify FEA automatic aerosolization points, fine-tune deployment plan. (EHA, WIV, NWHC, Duke-NUS, PARC, UNC). ***Subtask 5.2*** Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months before deployment proof-of-concept experiment (EHA Consultant Zhu, WIV). ***Subtask 5.3*** Run deployment experiment of most effective immune boosting molecules and delivery techniques via FEA aerosolization mechanism at one test and two control bat cave sites in Yunnan, China (PARC, EHA, WIV) ***Subtask 5.4*** Conduct bat viral surveillance of one test-site cave and two control caves for 4 months after deployment. (EHA Consultant Zhu, WIV). ***Subtask 5.5*** Assess efficacy of proof-of-concept trial (EHA, UNC, DNUS).
Milestone(s): Specific sites identified; Initiation and completion of trial under subtask 5.2-5.5.
Deliverables: Baseline immunology and viral shedding data from study populations. Proof-of-concept of deploying biological intervention. Post-deployment metrics for immune modulation and viral shedding in study populations. Report on proof-of-concept efficacy.

Section II **H. SCHEDULE AND MILESTONES**

See Table 2 Schedules and Milestones.

Section II **I. PREEMPT TRANSITION PLAN**

Technology from this project will be transitioned to multiple potential users throughout both phases. Partners PARC and the USGS National Wildlife Health Center will initiate planning for transition of aerosol deployment equipment within 12 months of Phase 1 including government customers such as DoD and USGS (for bat-related disease control) and possible manufacturers. IP rights for these efforts will be negotiated with DARPA. Prior to the completion of Phase 1, panels of fully sequence new viruses, in-silico models for pandemic prediction, and animal models that could be used to evaluate therapeutics will be shared with DARPA and collaboratively agreed upon for early distribution to DoD users such as DoD medical community, other UGS agencies, and ultimately made publically available. We have no plans to patent or otherwise restrict IP on this information unless requested by DARPA or requested by a project partner and approved by DARPA. Proposed technology to be experimentally deployed and evaluated in Phase 2 will be shared with DARPA and collaboratively agreed upon for early distribution to DoD users such as DoD medical community, other UGS agencies. We have no plans to patent or otherwise restrict IP on this information unless requested by DARPA or requested by a project partner and approved by DARPA.

PARC as a private industry partner (large business) is a fully-owned subsidiary of Xerox Corporation and is committed to commercializing the FEA technology through IP licensing for different applications spaces to different commercial partners. PARC has been and will continue to engage potential licensees (OEMs) in the biotechnology and biomedical fields for eventual transitioning of targeted delivery technology potentially developed in DEFUSE. PARC already has existing networks of business relations in the biotechnology and biomedical space, both large companies (Fortune 500, Fortune 1000) and small businesses and start-ups who could be transition partners for FEA as a wide-scale, large-area drug delivery device. In addition, in

Table 2. Schedule & Milestones

	Task #	Task Name/*Milestones*	Organization	Phase I Y1				Phase I Y2				Phase II OY1				OY2	
				Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2
TA1 Phase I	PI-Task 1	**Longitudinal sampling and surveillance**	EHA														
	1.1	IACUC and ACURO approval / *Permits obtained*	EHA														
	1.2	Invasive and non-invasive monthly specimen collection / *>3,000 specimens collected*	EHA (Zhu)														
	1.3	Bat connectivity and roost fidelity study / *All bats PIT tagged*	EHA (Zhu)														
	1.4	Assess bat home ranges / *Bat transmitters launched; data collected*	EHA (Zhu)														
	1.5	Real-time bat population monitoring / *IR cameras installed; data collected*	EHA (Zhu)														
	1.6	Develop and maintain project database / *Database built & secure; field data entered*	EHA														
	PI-Task 2	**Construct spillover risk models**	EHA														
	2.1	Joint species distribution models / *Models fit for Asian bat spp.; cave-level predictions*	EHA														
	2.2	Host and ecological machine learning models / *Models developed; per-species spillover risk*	EHA														
	2.3	Viral richness estimator model / *Models developed and refined w longitudinal surveillance data*	EHA														
	2.4	Prototype of 'spatial viral spillover risk' app / *Prototype app developed and beta-tested*	EHA														
	PI-Task 3	**Laboratory analysis of SARSr-CoV QS**	WIV														
	3.1	PCR screening of bat specimens / *Prevalance estimates; initial CoV seq data generated*	WIV														
	3.2	Sequence SARSr-CoV spike proteins / *Genetic sequences generated and made available*	WIV														
	3.3	Develop recombinant SARSr-CoV viruses / *Novel recombinants developed and recovered*	DNUS														
	3.4	Assess SARSr-CoV via deep sequencing / *Full genome data generated and made available*	UNC														
	PI-Task 4	**Experimental assays of SARSr-CoV QS**	UNC														
	4.1	Pre-screen SARSr-CoV via viral traits / *List of pre-screened QS using protein models and mutations*	UNC														
	4.2	SARSr-CoV chimeric virus testing in cell lines / *In vitro cell line experiments completed; data available*	UNC														
	4.3	In vivo pathogenesis in hACE2 transgenic mice / *Mouse experiments completed; data available*	UNC														
	4.4	Validate chimeric viruses by with full-genome QS / *Full genomes sequenced and made available*	UNC														
	4.5	Test synthetic modifications of spike proteins / *Experimental tests completed; data available*	UNC														
	4.6	Test effects of low-abundance, high consequence micro-variations on jump potential / *Variants quantified*	UNC														
	PI-Task 5	**Build and test Bayesian genotype-phenotype network models**	EHA														
	5.1	Use prior data to guide GS selection for characterization / *Prelim model developed; source code available*	EHA														
	5.2	Update model predictions using real-time data / *Model refined and validated w experimental data*	EHA														
	PI-Task 6	**Validate model predictions using human spillover evidence**	WIV														
	6.1	Design LIPS assays to SARS-CoV we have characterized / *Novel LIPS assays developed*	WIV														
	6.2	Deterimine LIPS assays specificity / *Specificity tested in rabbit model and data made available*	WIV														
	6.3	Validate LIPS assays using positive laboratory samples / *Assays validated and data made available*	WIV														
	6.4	Test previously-collected human sera for SARSr-CoV spillover / *Previous-collected human sera screened*	WIV														
	6.5	Validate BNM predictions using bat-human contact data / *Bayesian network model externally validated*	EHA														
TA2 Phase I	PI-Task 7	**Testing of broadscale immune boosting approach**	DNUS														
	7.1	Block bat-specific IFN negative regulators / *Experiments completed; data made available*	DNUS														
	7.2	Activate dampened bat-specific innate immune pathways / *Experiments and immune profiling completed*	DNUS														
	7.3	Test broadscale immune boosting in bat-mouse model / *Experiments completed; data made available*	DNUS														
	7.4	Test broadscale immune boosting in Eonycteris sp. / *Experiments completed; data made available*	DNUS														
	7.5	Test broadscale immune boosting in Rhinolophus sp. / *Experiments completed; data made available*	WIV														
	PI-Task 8	**Testing of targeted immune boosting approach**	UNC														

	8.1	Develop chimeric SARSr-CoV S immunogens / *Chimeras developed and made available for experiments*	UNC
TA2 Phase I	8.2	Test chimeric immunogens in humanized mice / *Experiments completed; data made available*	UNC
	8.3	Create raccoon poxvirus vectored immune boosting approach / *Experimental validation complete*	NWHC
	8.4	Test targeted immune boosting in Eonycteris sp. / *Experiments completed; data made available*	DNUS
	8.5	Test targeted immune boosting in Rhinolophus spp. / *Experiments completed; data made available*	WIV
	PI-Task 9	**Develop transcutaneous delivery methods**	UNC, DNUS, NWHC
	9.1	Synthesize Ac-DEX MPs / *Microparticles synthesized w immune boosting molecules*	UNC, DNUS
	9.2	Test MP metrics in vitro and in rodents / *Experiments completed in rodents; data made available*	UNC, DNUS
	9.3	Test MB safety in bats in WI and Singapore / *Safety experiments completed in bats; data made available*	NWHC, DNUS
	PI-Task 10	**Develop delivery systems for immune boosting molecules**	NWHC, PARC
	10.1	Trial RB-marked substances on captive bats / *Experiments completed in captive bats; data available*	NWHC
	10.2	Trial RB-marked substances on wild bats / *Experiments completed in wild bats; data available*	NWHC
	10.3	Develop prototype FEA device / *Prototype FEA device developed to deploy immune boosting molecules*	PARC
	10.4	Trial FEA device using RB on US captive bats / *Prototype FEA device tested on captive bats*	NWHC
TA1 Phase II	**PII-Task 1**	**Update 'spatial viral spillover risk' app**	EHA
	1.1	Incorporate laboratory and field results / *Bat species risk metrics from lab and field incoportated to model*	EHA
	1.2	Incorporate spatial and host-pathogen characteristics to risk ranking / *Models and source code updated*	EHA
	1.3	Link host species to viral diversity data / *Viral data integrated into host-pathogen spillover models*	EHA
	PII-Task 2	**Build and test Bayesian genotype-phenotype network models**	EHA
	2.1	Estimate intra- and inter-species mutation and recombination rates / *Recombination analyses completed*	EHA
	2.2	Simulate forward evolution to predict future and unsampled QS / *List of predicted future QS variants*	EHA
	2.3	Make predictions of likely future high-risk QS spillover / *High-risk SARSr-CoV QSs identified by model*	EHA
TA2 Phase II	**PII-Task 3**	**Develop delivery systems for immune boosting molecules**	PARC, NWHC
	3.1	Design and optimize facility for FEA prototype / *Develop motion- and time- actuated delivery system*	PARC
	3.2	Conduct field trials of RB on US wild bats / *Field trials completed on wild bats; data available*	PARC, NWHC
	PII-Task 4	**Build and test models to optimize deployment strategy**	EHA
	4.1	Develop robust stochastic SIR process model / *Model developed including bat contact rates*	EHA
	4.2	Fit SIR model to sampling data / *Fit SIR model using markov process; temporal cross-validation*	EHA
	4.3	Simulate deployment scenarios / *Develop scenario model simulations to optimize deployment*	EHA
	4.4	Test robustness of deployment strategies / *Various deployment scenarios tested w cave-specific data*	EHA
	PII-Task 5	**Demonstrate accuracy of delivery models and deploy methods**	All Orgs
	5.1	Identify specific sites and fine tune deployment plan / *Identify specific sites wihtin caves for deployment*	All Orgs
	5.2	Conduct baseline viral surveillance pre deployment / *Complete 4 month pre-deployment surveillance*	EHA, WIV
	5.3	Run deployment experiment / *Deploy immune boosting intervention at one test cave site and two controls*	EHA (Zhu), NHWC, PARC
	5.4	Conduct viral surveillance post deployment / *Complete 4 month post-deployment surveillance*	EHA, WIV
	5.5	Assess efficacy of proof-of-concept trial / *Data analyzed; including pre- and post- lab screening data*	EHA, UNC, DNUS

collaboration with our extended network of DEFUSE partners and with DARPA, we will further identify existing government needs for our delivery technology, particularly in wildlife health management (in collaboration with EHA and USGS-NWHC) and in suppression of emerging threats (in collaboration with government agencies such as the CDC). PARC will leverage this knowledge in developing a needs-based commercialization plan with potential partners.

Project DEFUSE partners come from academic, government, private industry, private non-profit institutions and will develop a coherent transition plan for research findings, data and any technology developed in this work.

Section II J. PREEMPT RISK MITIGATION PLAN

Risks: Personnel safety, biosafety, mitigation of risks to public health and animal safety

Animal Use & safety: All work with wild bats will be conducted in China by EcoHealth Alliance staff and Wuhan Institute of Virology. Capture and sampling techniques have been previously approved by Tufts University School of Veterinary Medicine IACUC under our NIH NIAID award (Daszak, PI). Experimental work using bats and or transgenic mice will be conducted at the BSL-3 lab in WIV, Duke-NUS, UNC, or NWHC. Each partner institute will apply for and procure animal research approval from its respective IACUC. All animal work conducted by EcoHealth Alliance in China will be overseen by both the IACUC at WIV and the IACUC at Tufts. Each partner institute will be responsible for ensuring the training and safety of its laboratory personnel, which will be documented by EcoHealth Alliance, and each partner has extensive experience and a record of safety with the techniques and procedures for lab animal experiments described in this protocol. *Field safety:* Free-ranging bats will be captured using either a mist net or harp trap. The net system is manned by two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are placed into a small cloth bag and hung from a branch or post until samples are collected. Bats are held for a maximum of six hours. Field personnel will be required to conduct a hazard assessment prior to each field sampling period and at each location. *Biosafety and Personal Protective Equipment*: Dedicated clothing will be worn in the field which provides protection against injury from bites or scratches as well as nitrile gloves (double layer), an N95 respirator, and safety glasses or a face shield when removing bats from a net or trap, and when sampling bats. External clothing and all equipment will be decontaminated at the field site using virkon, and biohazardous waste will be contained in biohazard bags and sharps containers and incinerated at WIV or Yunnan CDC facilities. Personnel will wear water-impermeable Tyvek suits, rubber boots, and powered air purifying respirators (PAPRs) when entering a cave for sample collection or image collection (e.g. LiDAR) and will doff and dispose of PPE and disinfect PAPRs on exiting the cave. **All field personnel will be immunized against rabies and demonstrate a current (within 6 months) protective titer according to CDC guidelines[132].** Personnel without PPE training and complete rabies immunization will not be permitted to work with bats or enter the study caves. Use of LiDAR to map study caves will be conducted by trained personnel with caving experience.

Risks to general public: The proposed work has minimal risk to the general public, as sampling will be done near the cave sites and not in populous areas. Our team has extensive experience

Section II K. ETHICAL, LEGAL, SOCIETAL IMPLICATIONS

All activities in this project will be done with strict adherence to US and Chinese law, with permission from the Chinese government and local authorities to conduct field work. We will conduct educational outreach to local wildlife authorities and cultural leaders so that there is a public understanding of what we are doing and why we are doing it, particularly because of the common practice of bat consumption in the region (**see also Risk Mitigation Strategy**). These agents have not been tested on humans, but they have been shown to be safe in a variety of lab animal models. There is minimal risk associated with human exposure to the fluid containing MPs. We will explain the risk mitigation strategies and safety data that were considered when developing this study, and how this could be of benefit to local communities. There is also a potential benefit to local communities if the agents are effective in reducing viral shedding. The broader societal impact of this project could be significant, as wildlife immunization against viral zoonoses has been limited to date. However, this may open up a field where animal reservoirs for known high-risk agents could be "immunized" at high risk times of year which could reduce the number or magnitude of human outbreaks. This would add a valuable countermeasure to ecological studies that have elucidated the timing of viral spillover from animal reservoirs to human or livestock populations. We will develop a plan to ensure that the details of technologies developed and tested for deploying biological immune modulating agents are made available publicly so that they can be adapted to other types of medical interventions and pathogens. There may be conservation benefits, where wildlife reservoirs are considered less threatening to public health and therefore there may be less impetus to exterminate or extirpate local populations as a public health measure – particularly if an option to reduce the risk of spillover through a wildlife vaccination effort is available.

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Section III RELEVANT PAPERS

We are attaching three relevant papers:

- Ge et al. (2017). Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 Receptor. *Nature*.
- Menachery et al. (2016). SARS-like WIV-CoV poised for human emergence. *PNAS*.
- Zhou et al. (*In Press*). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature*.